

10/08/99
1c712 U.S. PTO

A

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Dkt. No.: UTXC:528--1

Prior Application Examiner:
Levy, N.

Assistant Commissioner for Patents
Washington, D.C. 20231

Classification Designation:
424-405.000

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09/415890
JC675 U.S. PTO

Prior Group Art Unit: 1617

| | |
|--------------------------------|-----------------|
| CERTIFICATE OF EXPRESS MAILING | |
| NUMBER | EL258326531US |
| DATE OF DEPOSIT | October 8, 1999 |

REQUEST FOR FILING CONTINUATION APPLICATION
UNDER 37 C.F.R. § 1.53(b)

This is a request for filing a continuation application under Rule 53(b) (37 C.F.R. § 1.53(b)) of co-pending prior application Serial No. 08/911,607 filed August 15, 1997, entitled "PARENTERAL PIMARICIN AS TREATMENT OF SYSTEMIC INFECTIONS."

- ☒ 1. Enclosed is a copy of the prior application Serial No. 08/911,607 as originally filed, including specification, claims, drawings, and declaration. The undersigned hereby verifies that the attached papers are a true copy of the prior application as originally filed and identified above, that no amendments (if any) referred to in the declaration filed to complete the prior application introduced new matter therein, and further that this statement was made with the knowledge that willful

false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statement may jeopardize the validity of the application or any patent issuing thereon.

(a) ☐ The inventorship is the same as prior Application Serial No. 08/911,607.

(b) ☒ Deletion of inventor(s). Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. § 1.63(d)(2) and 1.33(b).

(c) ☐ Priority of foreign patent application number , filed in
is claimed under 35 U.S.C. § 119(a)-(e). The certified copy:

☐ is enclosed.

☐ has been filed in the prior Application Serial No.

- ☒ 2. Enclosed is a check in the amount of \$695.00 to cover the filing fee as calculated below and the fee for any new claims added in the Preliminary Amendment referred to in Clause No. 7 below.

CLAIMS AS FILED IN THE PRIOR APPLICATION
LESS CLAIMS CANCELED BELOW

| FOR | NUMBER FILED | NUMBER EXTRA | RATE | FEE |
|-----------------------------------|-----------------|-----------------|-----------|----------|
| Basic Fee ----- | | | | \$380.00 |
| Total Claims | 55 - 20 = | 35 X | \$9.00 = | \$315.00 |
| Independent Claims | 3 - 3 = | 0 X | \$39.00 = | \$0.00 |
| Multiple Dependent Claim(s) ----- | | | | \$-0.00 |

TOTAL FILING FEES:

| FOR | NUMBER FILED | NUMBER EXTRA | RATE | FEE |
|-----|-----------------|-----------------|------|-----------------|
| | | | | \$695.00 |

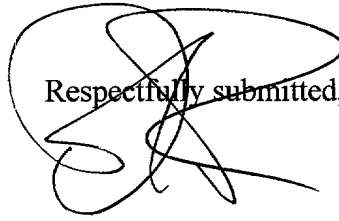
- ☒ 3. Applicant is entitled to Small Entity Status for this application.
- ☒ (a) A small entity statement is enclosed.
- ☒ (b) A small entity statement was filed in the prior nonprovisional application and such status is still proper and desired.
- ☐ (c) Small entity status is no longer claimed.
- ☒ 4. If the check is missing or insufficient, the Assistant Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§ 1.16 to 1.21 which may be required for any reason relating to this application, or credit any overpayment to Arnold White & Durkee Deposit Account No. 01-2508/UTXC:528--1/PAR.
- ☐ 5. Enclosed is a copy of the current Power of Attorney in the prior application.
- ☒ 6. Address all future communications to:

David L. Parker
ARNOLD WHITE & DURKEE
P.O. Box 4433
Houston, Texas 77210
(512) 418-3000

- ☒ 7. The prior application is presently assigned to Board of Regents, The University of Texas System.
- ☒ 8. Enclosed is a preliminary amendment. Any additional fees incurred by this amendment are included in the check at No. 2 above and said fee has been calculated after calculation of claims and after amendment of claims by the preliminary amendment.
- ☐ 9. Cancel in this application claims of the prior application before calculating the filing fee. (At least one original independent claim must be retained).
- ☒ 10. Amend the specification by inserting before the first line the sentence: --This is a continuation of co-pending application Serial No. 08/911,607 filed August 15, 1997--.
- ☐ 11. Enclosed are formal drawings.
- ☐ 12. An Information Disclosure Statement (IDS) is enclosed.
- ☐ (a) PTO-1449.
- ☐ (b) Copies of IDS citations.
- ☐ 13. Transfer the sequence information, including the computer readable form previously submitted in the parent application, Serial No. filed , for use in this application. Under 37 C.F.R. § 1.821(e), Applicant states that the paper copy of the sequence listing in this application is identical to the computer readable copy in parent application Serial No. filed . Under 37 C.F.R. § 1.821(f), Applicant also states that the information recorded in computer readable form is identical to the written sequence listing.
- ☒ 14. Other: Exhibit A

- ☒ 15. Return Receipt Postcard (should be specifically itemized).

Respectfully submitted,



David L. Parker
Reg. No. 32,165
Attorney for Applicant

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P.O. Box 4433
Houston, Texas 77210-4433
(512) 418-3000

Date: October 8, 1999

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Borje S. Andersson et al

Serial No.: 08/911,607

Filed: August 15, 1997

For: PARENTERNAL PIMARICIN AS
TREATMENT OF SYSTEMIC
INFECTIONS

Group Art Unit: 1615

Examiner: Levy, N.

Atty. Dkt. No.: UTXC528

DECLARATION CLAIMING SMALL ENTITY STATUS
37 C.F.R. §§ 1.9(F) AND 1.27(D) - NONPROFIT ORGANIZATION

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: Board of Regents, The University of Texas System

Address of Organization: 201 West Seventh Street
Austin, Texas 78701

The type of organization is a university.

I hereby declare that the organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. § 1.9(e) (1), and thus is a "small entity" as defined in § 1.9(f), for purposes of paying reduced fees under Sections 41(a) and (b) of Title 35, United States Code, with regard to the above-referenced application.

I hereby declare that exclusive rights to the invention have been conveyed to and remain with the organization, with respect to the above-referenced invention, nor have I assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an

independent inventor under 37 CFR § 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR. § 1.9(d) or a nonprofit organization under 37 CFR § 1.9(e), with the exception that the Government may have rights in the invention pursuant to a funding agreement under 35 U.S.C. § 202(c)(4): None

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

BOARD OF REGENTS, THE UNIVERSITY OF
TEXAS SYSTEM

By:  _____

Name: Ray Farabee

Title: Vice Chancellor and General Counsel

Date: 9/14/98

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Borje S. Andersson

Serial No.: Unknown

Filed: Unknown

For: PHARMACOLOGICALLY
ACCEPTABLE SOLVENT VEHICLES

Group Art Unit: Unknown

Examiner: Unknown

Atty. Dkt. No.: UTXC:528--1/PAR

CERTIFICATE OF EXPRESS MAILING

NUMBER EL258326531US

DATE OF DEPOSIT October 8, 1999

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Please amend this application as follows:

In The Specification:

At page 1, please replace the existing title of the application with the following title: --
PHARMACOLOGICALLY ACCEPTABLE SOLVENT VEHICLES --.

At page 2, line 1, insert the following:

--This is a continuation of co-pending application Serial No. 08/911,607, filed August 15,
1997.--

In the Claims

Cancel all the pending claims, claims 1-15, 24 and 25, without prejudice.

Add claims 26-80 as follows:

--26. A solvent vehicle, comprising a pharmaceutically acceptable dipolar aprotic solvent and a pharmaceutically acceptable aqueous secondary solvent.

27. The composition of claim 26, wherein said aprotic solvent comprises N,N-dimethylacetamide, castor oil, dimethylsulfoxide, 1,2,-propylene-diol, glycerol or polyethylene glycol-400.

28. The solvent vehicle of claim 27, wherein said aprotic solvent comprises N,N-dimethylacetamide.

29. The solvent vehicle of claim 27, wherein said aprotic solvent comprises castor oil.

30. The solvent vehicle of claim 27, wherein said aprotic solvent comprises dimethylsulfoxide.

31. The solvent vehicle of claim 27, wherein said aprotic solvent comprises 1,2,-propylene-diol.

32. The solvent vehicle of claim 27, wherein said aprotic solvent comprises glycerol.

33. The solvent vehicle of claim 27, wherein said aprotic solvent comprises polyethylene glycol-400.

34. The composition of claim 26, wherein said secondary solvent comprises aqueous lipid emulsion, water, saline solution, dextrose solution, glacial acetic acid, lipid solution or parenteral infusion fluids.

35. The solvent vehicle of claim 34, wherein said secondary solvent comprises an aqueous lipid emulsion.

36. The solvent vehicle of claim 35, wherein said aqueous lipid emulsion comprises Liposyn II™.

37. The solvent vehicle of claim 35, wherein said aqueous lipid emulsion comprises an aqueous soy bean lipid emulsion.

38. The solvent vehicle of claim 37, wherein said aqueous soy bean lipid emulsion comprises Intralipid™.

39. The solvent vehicle of claim 35, wherein said aqueous lipid emulsion comprises a lipid component that includes at least one vegetable oil and at least one fatty acid.

40. The solvent vehicle of claim 39, wherein said lipid component comprises at least about 5% by weight soybean oil and at least about 50% by weight fatty acids.

41. The solvent vehicle of claim 34, wherein said said secondary solvent comprises water.

42. The solvent vehicle of claim 34, wherein said secondary solvent comprises saline solution.

43. The solvent vehicle of claim 34, wherein said secondary solvent comprises dextrose solution.

44. The solvent vehicle of claim 43, wherein said dextrose solution comprises 5% to 70% dextrose in water.

45. The solvent vehicle of claim 44, wherein said dextrose solution comprises 5% or 10% dextrose solution.

46. The solvent vehicle of claim 34, wherein said secondary solvent comprises glacial acetic acid.

47. The solvent vehicle of claim 26, wherein said secondary solvent comprises a lipid solution.

48. The solvent vehicle of claim 26, wherein said secondary solvent comprises parenteral infusion fluids.

49. The solvent vehicle of claim 26, wherein said composition further comprises an active agent, a drug, pharmaceutically acceptable carriers, adjuvants or biologically active substances.

50. The solvent vehicle of claim 26, wherein said solvent vehicle comprises anhydrous N,N-dimethylacetamide and polyethylene glycol-400.

51. The solvent vehicle of claim 26, wherein said solvent vehicle comprises glacial acetic acid and polyethylene glycol-400.

52. The solvent vehicle of claim 26, wherein said solvent vehicle comprises anhydrous N,N-dimethylacetamide and aqueous lipid.

53. The solvent vehicle of claim 52, wherein said aqueous lipid is Intralipid™.

54. The solvent vehicle of claim 53, wherein said solvent vehicle comprises anhydrous N,N,-dimethylacetamide and Intralipid™ in a 1:10 volume ratio.

55. The solvent vehicle of claim 53, wherein said solvent vehicle comprises anhydrous N,N,-dimethylacetamide diluted with 9 volumes 20% Intralipid™.

56. The solvent vehicle of claim 53, wherein said solvent vehicle further comprises normal saline or 5% dextrose solution.

57. The solvent vehicle of claim 26, wherein said solvent vehicle comprises anhydrous N,N-dimethylacetamide, polyethylene glycol-400 and 1,2-propylene diol.

58. The solvent vehicle of claim 26, wherein said solvent vehicle comprises anhydrous N,N-dimethylacetamide, polyethylene glycol-400, 1,2-propylene diol and dimethylsulfoxide.

59. The solvent vehicle of claim 58, wherein said solvent vehicle comprises anhydrous N,N-dimethylacetamide, polyethylene glycol-400, 1,2-propylene diol and dimethylsulfoxide in equal volume ratios.

60. The solvent vehicle of claim 26, wherein said vehicle comprises glacial acetic acid, and wherein said vehicle further comprises anhydrous N,N-dimethylacetamide, dimethylsulfoxide or Intralipid™.

61. The solvent vehicle of claim 26, wherein said solvent vehicle comprises glacial acetic acid, dimethylsulfoxide and Intralipid™.

62. The solvent vehicle of claim 61, wherein said solvent vehicle comprises glacial acetic acid, dimethylsulfoxide, and Intralipid™ in a 2:6:3 volume ratio.

63. The solvent vehicle of claim 26, wherein said composition is administered to an animal.

64. The solvent vehicle of claim 26, wherein said composition is administered to a human.

65. The solvent vehicle of claim 26, wherein said composition is administered by parenteral injection.

66. The method of claim 65, wherein said parenteral injection is intravascular or intravenous injection.

67. The solvent vehicle of claim 26, wherein said composition is administered as an aerosol.

68. The solvent vehicle of claim 26, wherein said vehicle is lyophilized.

69. A composition, comprising a drug, a pharmaceutically acceptable dipolar aprotic solvent and a pharmaceutically acceptable aqueous secondary solvent.

70. The composition of claim 69, wherein said aprotic solvent comprises N,N-dimethylacetamide, castor oil, dimethylsulfoxide, 1,2,-propylene-diol, glycerol or polyethylene glycol-400.

71. The composition of claim 69, wherein said secondary solvent comprises aqueous lipid emulsion, water, saline solution, dextrose solution, glacial acetic acid, lipid solution or parenteral infusion fluids.

72. The composition of claim 69, wherein said composition has been lyophilized.

73. The composition of claim 69, wherein said composition further comprises a pharmaceutically acceptable aqueous solvent.

74. The composition of claim 73, wherein said aqueous solvent comprises a parenteral infusion fluid.

75. The composition of claim 74, wherein said parenteral infusion fluid is saline solution, dextrose solution or distilled water.

76. The composition of claim 73, wherein said aqueous solvent is suitable for parenteral administration to a mammal.

77. The composition of claim 76, wherein said mammal is a human.

78. A composition, comprising a pharmacologically active agent, a pharmaceutically acceptable dipolar aprotic solvent and a pharmaceutically acceptable aqueous secondary solvent.

79. The composition of claim 78, wherein said aprotic solvent comprises N,N-dimethylacetamide, castor oil, dimethylsulfoxide, 1,2,-propylene-diol, glycerol or polyethylene glycol-400.

80. The composition of claim 78, wherein said secondary solvent comprises aqueous lipid emulsion, water, saline solution, dextrose solution, glacial acetic acid, lipid solution or parenteral infusion fluids.--

REMARKS

Status of the Claims:

All the previously pending claims, claims 1-15, 24 and 25, have been cancelled without prejudice or disclaimer. Claims 26-80 have been added. Claims 26-80 are presently pending in the case.

For the convenience of the examiner, the presently pending claims are included herein as

Exhibit A.

Addition of Claims:

Applicant now elects to prosecute in the present continuation application subject matter described in co-pending Patent Application Serial No. 08/911,607 but not previously claimed. Copending Application Serial No. 08/911,607 describes and presently claims antifungal compositions comprising pimaricin, dipolar aprotic solvent and an aqueous secondary solvent. However, the specification also discloses novel vehicles or "solvent vehicles", and Applicant presently submits claims to compositions comprising the disclosed solvent vehicles. Written support for the term "solvent vehicles" may be found throughout the specification as filed, particularly at page 9, lines 17-18 and at page 18, lines 10-11. Support for the embodiment of the novel solvent vehicles are described throughout the specification and claims as filed, particularly at page 14, lines 22-29:

The objectives of this experiment were to: (1) design stable pimaricin formulations that are suitable for parenteral administration; (2) establish the chemical and physical stability of pimaricin **in the novel vehicles**; (3) establish the solubility of pimaricin in these vehicles when mixed with NS, dextrose in water, and Intralipid™; and (4) investigate the *in vitro* properties of these

formulations; i.e. their osmolality, hemolytic potential, and cytotoxicity, to show that they are appropriate for the intended purpose. (emphasis added).

Various specific examples of solvent vehicles are described in the specification as filed, such as at page 16, lines 14-19; at page 17, lines 4-6 and 20-28; at page 19, line 28 to page 20, line 13, including Table 2; at page 21, lines 2-11; at page 23, Table 4 and lines 4-5; and at Figures 6-8. For the convenience of the Examiner, the relevant text, at page 19, line 28 to page 20, line 13, describing examples of various vehicles is shown below:

It is desirable that a parenteral formulation of a pharmacologically active agent be isosmotic to blood. A hypertonic delivery system can be utilized if the drug/solvent is infused through a (central) venous catheter and gradually diluted in a large blood volume. The osmotic pressure of the various formulations is shown in Table 2.

Table 2

Osmotic Pressures of Various Vehicles with and without Pimaricin

| Solution | N | Osmotic pressure mOsm/kg |
|---|---|-----------------------------|
| Water | 3 | 3 |
| Normal saline | 3 | 233 |
| 5% dextrose in water | 3 | 286 |
| Blood, human | 6 | 280-295 |
| DMA:PEG:PG | 3 | 4492 |
| Pimaricin in DMA:PEG:PG | 3 | 4732 |
| Intralipid | 3 | 340 |
| DMA:Intralipid (1:10, v/v) | 3 | 2067 |
| Pimaricin in DMA:Intralipid (1:10, v/v, fresh) | 3 | 1930 |
| DMA:Intralipid (1:10, lyophil.-reconstit.) | 3 | 157 |
| Pimaricin (1 mg/ml) in DMA:Intralipid (1:10, lyophil.-reconstit.) | 3 | 208 |
| Pimaricin (25 mg/ml) in DMA:Intralipid (1:10, lyophil.-reconstit.) | 3 | 243 |

("n" represents the number of independent determinations.)

The DMA-stock formulation with or without pimaricin was very hypertonic; its osmotic pressure was more than 1,900 mOsm/kg, as compared with 280-295 mOsm/Kg for human blood. The DMA/PG/DMSO/PEG and DMA/PEG solvents were almost as hypertonic. In contrast, the DMA/Intralipid preparation was closer to isosmotic when reconstituted after lyophilization. Similarly, the

lyophilized/reconstituted HAc/DMSO/Intralipid™ vehicle was also close to isosmotic. Adding pimaricin to the respective vehicles did not appreciably change their osmolarity ($P > 0.05$).

Specific examples in the novel vehicles, either alone or in combination with other drugs, are also described in the specification as filed, such as at page 9, lines 12-17:

We have investigated N,N-dimethylacetamide (DMA), DMSO, glycerol, 1,2,-propylene-diol (PG), and polyethylene glycol-400 (PEG) as primary solvents that would be miscible in secondary solvents, examples of which are normal saline, dextrose in water (5% or 10%), and an aqueous soy bean lipid emulsion (Intralipid™). These solvents are examples of vehicles in which pimaricin could be suitably solubilized, yet be safe for human administration, **alone or in combinations with other drugs**. (Emphasis added).

Thus, the disclosures of the solvent vehicles are not limited to those that comprise pimaricin, but also include other drug/vehicle combinations, as described at page 19, line 27 to page 20, line 2:

It is desirable that a parenteral formulation of a **pharmacologically active agent** be isosmotic to blood. A hypertonic delivery system can be utilized if the **drug/solvent** is infused through a (central) venous catheter and gradually diluted in a large blood volume. (Emphasis added).

The novel solvent vehicles can also comprise various other components, as described at page 32, lines 24-25:

Compositions of the present invention can further include additional pharmaceutically acceptable carriers, adjuvants, and/or biologically active substances.

However, these disclosures of the novel solvent vehicles in combination with other materials are not limited to combinations with an active agent, drug, pharmaceutically acceptable carriers, adjuvants or biologically active substances, as described at page 33, line 8-11:

The preceding description of specific embodiments of the present invention is not intended to be a complete list of every possible embodiment of the invention. Persons skilled in this field will recognize that modifications can be made to the specific embodiments described here that would be within the scope of the present invention.

Thus, the specification and originally filed claims provides ample written support for the added claims directed to compositions comprising solvent vehicles.

Accordingly, the specification has been amended to at page 1 to give a descriptive title of this aspect of the invention, and at page 2, line 1 to recite the relationship of this application with the parent case, Patent Application Serial No. 08/911,607.

The active claims in this case are claims 26-80.

Claims 26-67 have been added to described the embodiment of the invention, a "solvent vehicle." Support for these claims can be found throughout the specification as filed, with particular support found at page 16, lines 14-19; at page 17, lines 4-6 and 20-28; at page 19, line 28 to page 20, line 13, including Table 2; at page 21, lines 2-11; at page 23, Table 4 and lines 4-5; and at Figures 6-8.

Claims 26-28, 34-35 and 39-43 describe various embodiments of a solvent vehicle. Support for these claims can be found in the specification and claims as originally filed, with particular support found at originally filed claims 1-8 and 20-24; at page 13, lines 25-27, page 15, lines 2-3 and Table 1.

Claims 29-31 and 33 describe embodiments of the aprotic solvent. Support for these claims can be found throughout the specification as filed, with particular support found at page 13, lines 25-27, page 15, lines 2-3 and Table 1.

Claim 32 describe embodiments of the aprotic solvent. Support for this claim can be found throughout the specification as filed, with particular support found at page 9, lines 12-15.

Claim 36 describes an embodiment of an aqueous lipid emulsion. Support for this claim can be found throughout the specification as filed, with particular support found at 13, lines 2-5.

Claim 37 describes an embodiment of an aqueous lipid emulsion. Support for this claim can be found throughout the specification as filed, with particular support found at page 8, lines 21-22, at page 9, lines 12-14 and at page 13, lines 25-27.

Claim 38 describes an embodiment of an aqueous soy bean lipid emulsion. Support for this claim can be found throughout the specification as filed, with particular support found at page 8, lines 21-22, at page 9, lines 12-14 and at page 13, lines 25-27.

Claims 44 and 45 describe embodiments of the dextrose solution. Support for these claims can be found throughout the specification as filed, with particular support found at page 10, lines 19-21 and at page 9, lines 12-15, respectively.

Claim 46 describes an embodiment of the secondary solvent. Support for this claim can be found throughout the specification as filed, with particular support found at page 13, lines 25-28.

Claim 47 describes an embodiment of an aqueous lipid solution. Support for this claim can be found throughout the specification as filed, with particular support found at page 7, line 8 and page 10, line 30.

Claim 48 describes an embodiment of the second solvent. Support for this claim can be found throughout the specification as filed, with particular support found at page 5, lines 19-21 and page 12, line 21 to page 13, line 5.

Claim 49 describes an embodiment wherein the composition further comprises an active agent, drug, pharmaceutical acceptable carriers, adjuvants or biologically active substances. Support for this claim can be found throughout the specification as filed, with particular support found at page 9, lines 12-17; at page 19, line 27 to page 20, line 2; described at page 32, lines 24-25 and at page 33, lines 1-4.

Claims 50-62 describes specific solvent vehicle compositions. Support for these claims can be found throughout the specification as filed, with particular support found at page 6, line 1-8; at page 7, lines 11-19; at page 17, lines 20-28 and at page 23, lines 4-5 and Table 4; at page 19, line 28 to page 20, line 13, including Table 2; at page 21, lines 2-11; at page 23, Table 4 and lines 4-5; and at Figures 6-8.33-35, 40-45.

Claim 63 describes an embodiment wherein the composition is administered to an animal. Support for this claim can be found throughout the specification as filed, with particular support found at page 10, lines 8-12.

Claim 64 describes an embodiment wherein the composition is administered to a human. Support for this claim can be found throughout the specification as filed, with particular support found at page 10, lines 8-12.

Claim 65 describes an embodiment wherein the composition is administered by parenteral injection. Support for this claim can be found throughout the specification as filed, with particular support found at page 6, lines 5-8.

Claim 66 describes an embodiment wherein the parenteral injection is intravascular or intravenous. Support for this claim can be found throughout the specification as filed, with particular support found at page 32, lines 29-30 and at page 6, lines 1-4.

Claim 67 describes an embodiment wherein the composition is administered as an aerosol. Support for this claim can be found throughout the specification as filed, with particular support found at page 32, line 30 to page 33, line 1.

Claim 68 describes an embodiment wherein the composition has been lyophilized. Support for this claim can be found throughout the specification as filed, with particular support found at page 5, lines 12-30.

Independent claim 69 describes an aspect of the invention, wherein the composition comprises a drug, an aprotic solvent and a secondary solvent. Support for this claim can be found throughout the specification as filed, with particular support found at page 5, lines 12-16, at page 19, line 28 to page 20, line 2, and at originally filed claim 17.

Claim 70 describes various embodiments of the aprotic solvent. Support for this claim can be found in the specification and claims as originally filed, with particular support found at originally filed claims 1-8 and 20-24; at page 13, lines 25-27, page 15, lines 2-3, Table 1; and at page 9, lines 12-15.

Claim 71 describes various embodiments of the secondary solvent. Support for this claim can be found throughout the specification as filed, with particular support found at originally filed claims 1-8 and 20-24; at page 13, lines 25-27, page 15, lines 2-3 and Table 1.

Claim 72 describes an embodiment wherein the composition has been lyophilized. Support for this claim can be found throughout the specification as filed, with particular support found at page 5, lines 12-30.

Claim 73 describes an embodiment wherein the composition further comprises a pharmaceutically acceptable aqueous solvent. Support for this claim can be found throughout the specification as filed, with particular support found at page 5, lines 16-18.

Claim 74 describes an embodiment wherein the aqueous solvent is a parenteral infusion fluid. Support for this claim can be found throughout the specification as filed, with particular support found at page 5, lines 19-21.

Claim 75 describes an embodiment of particular types of parenteral infusion fluids. Support for this claim can be found throughout the specification as filed, with particular support found at page 5, lines 19-21.

Claim 76 describes an embodiment of the composition wherein it is suitable for parenteral administration to a mammal. Support for this claim can be found throughout the specification as filed, with particular support found at page 5, lines 16-18.

Claim 77 describes an embodiment wherein the mammal is a human. Support for this claim can be found throughout the specification as filed, with particular support found at page 5, lines 8-9.

Independent claim 78 describes an aspect of the invention, wherein the composition comprises a pharmacologically active agent, an aprotic solvent and a secondary solvent. Support for this claim can be found throughout the specification as filed, with particular support found at page 19, lines 27-28.

Claim 79 describes various embodiments of the aprotic solvent. Support for this claim can be found in the specification and claims as originally filed, with particular support found at originally filed claims 1-8 and 20-24; at page 13, lines 25-27, page 15, lines 2-3, Table 1; and at page 9, lines 12-15.

Claim 80 describes various embodiments of the secondary solvent. Support for this claim can be found in the specification and claims as originally filed, with particular support found at originally filed claims 1-8 and 20-24; at page 13, lines 25-27, page 15, lines 2-3 and Table 1.

It is believed that no fee is due; however, should any fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason, the Assistant Commissioner is authorized to deduct said fees from Arnold White & Durkee Deposit Account No. 01-2508/UTXC:528--1/PAR.

Respectfully submitted,

David L. Parker
Reg. No. 32,165
Attorney for Applicants

ARNOLD, WHITE & DURKEE
P.O. Box 4433
Houston, Texas 77210-4433
(512) 418-3000

Date: October 8, 1999

PATENT
UTXC528

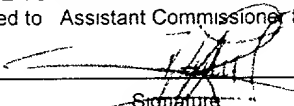
APPLICATION FOR UNITED STATES LETTERS PATENT

for

**PARENTERAL PIMARICIN AS TREATMENT OF SYSTEMIC
INFECTIONS**

by

Borje S. Andersson and Elias J. Anaissie

| EXPRESS MAIL MAILING LABEL | |
|---|------------------------|
| NUMBER | <u>EF943306906US</u> |
| DATE OF DEPOSIT | <u>August 15, 1997</u> |
| I hereby certify that this paper or fee is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C F R 1 10 on the date indicated above and is addressed to Assistant Commissioner for Patents, Washington D C. 20231. | |
|  _____ Signature | |

BACKGROUND OF THE INVENTION

The present invention relates to a drug formulation that is useful for the treatment and suppression of systemic infections, for example those caused by *Aspergillus* and *Fusarium* species.

Disseminated fungal infections constitute one of the most difficult challenges for clinicians caring for patients with hematological cancer (1). While the incidence of hematogenous candidiasis has been significantly reduced with the introduction of fluconazole prophylaxis, the opportunistic molds have become the leading cause of infectious mortality in this patient population (2). Aspergillosis clearly remains the most common mold infection in patients with hematological cancer. However, new opportunistic pathogens have now emerged as a cause of life-threatening infection worldwide. The most frequently reported of these pathogens is *Fusarium* (3-7). Infection with *Fusarium* is associated with a very high mortality and is typically refractory to amphotericin B. Since infection with this organism may mimic aspergillosis, patients are usually treated with Amphotericin B (AMB), an agent with poor activity against Fusariosis. In addition, the airways are the most common primary site of inoculation and infection and are almost always involved in disseminated disease (3-7). Hence, any drug with good activity against Fusariosis (particularly if it is also active against Aspergillosis) that could be given parenterally and also through aerosolization or nebulization will significantly improve our therapeutic armamentarium.

In addition to being ineffective against Fusariosis, Amphotericin B, the first-line treatment for documented or suspected systemic mold infections carries with it common (>75% of treated subjects), substantial and frequently dose-limiting nephrotoxicity, requiring at times hemodialysis. The acute infusion-related adverse events (severe shaking chills, fever, nausea, vomiting, headache) are quite troublesome to patients. Other serious side effects, such as cardiac arrhythmias, bone marrow suppression, neuropathies, and convulsions are also encountered with the use of AMB, although less frequently (8). The introduction of liposomally encapsulated AMB was anticipated to improve the control of systemic fungal infections (9,10). Its administration changed the drug's biodistribution, allowing significantly higher doses to be delivered with (hopefully) better anti-fungal

1 effects, without encountering serious nephrotoxicity (11-13). In spite of an increased renal
2 tolerance to liposomal AMB compared with the parent drug, this new formulation has
3 several limitations, including its high cost (presently around \$800 per day) which has
4 limited its use, its toxicity profile which is identical to that of Amphotericin B (except for
5 the kidney toxicity) and the fact that there is no evidence that this new drug formulation has
6 actually improved the ultimate control rate of serious mycotic/mold infections. Liposomal
7 AMB has recently received federal approval for routine clinical use in the U.S.

8 The only important clinically available alternative to AMB for the treatment of
9 systemic mold infections is itraconazole (Sporinox™) (13, 14, 15). This agent is presently
10 available exclusively as an oral preparation that is only erratically absorbed from the
11 intestinal tract, yielding variable plasma concentrations with highly unpredictable anti-
12 fungal activity (13) and has little or no activity against *Fusarium*. This bioavailability
13 problem is particularly difficult to manage in bone marrow transplant (BMT) patients who
14 are at highest risk for invasive mold infections. Such patients typically have severe
15 mucositis that interferes with their ability to swallow the itraconazole capsule and also
16 impairs the already erratic intestinal absorption of the drug. In addition, these patients
17 commonly receive antacids or H2 blockers, both agents known to interfere with the
18 absorption of itraconazole.

19 Based on the above considerations, the development of an effective antimycotic
20 agent with low normal organ toxicity, high bioavailability, predictable pharmacokinetics
21 after parenteral administration, and activity against both *Fusarium* and *Aspergillus* appears
22 highly desirable. Pimaricin, or natamycin (Fig. 1) would fulfill the criterion of being an
23 effective anti-fungal agent, exerting significant activity against molds, particularly *Fusarium*
24 and *Aspergillus*. It was first isolated in 1955 from a strain of *Streptomyces* (15). Pimaricin
25 exhibited a wide range of *in vitro* activity against fungi, yeast, and trichomonads (15, 16,
26 17). The drug was found to have little or no toxicity after oral administration, being
27 virtually non-absorbable from the gastrointestinal tract (16, 17). However, the lack of
28 solubility of pimaricin in various solvents, both aqueous and organic, compatible with
29 human administration has severely restricted its use in clinical medicine. Pimaricin's
30 medical utilization is currently confined to the topical treatment of corneal fungal infections

(18) and the prevention of such infections in contact-lens users. In contrast, pimarin's prominent chemical stability paired with its apparent lack of intestinal absorption and systemic toxicity formed the basis for its FDA-approved use in the food industry, where it is used to prevent the proliferation of (aflatoxin-producing) molds (19).

A parenterally acceptable, nontoxic formulation of pimarin would be potentially beneficial not only for cancer patients, but also for other groups of immunocompromised patients, e.g. those suffering from HIV and those having recently undergone open heart surgery, all of which are commonly targets for opportunistic infections.

Past attempts to solubilize pimarin in vehicles that are safe for intravascular administration in humans have all failed, despite the hard work by Stuyk and others (15, 16, 17). Thus, Korteweg and coworkers attempted to solubilize the drug by mixing it with a complex polysaccharide (16). Although the water-solubility of this formulation increased dramatically, its antifungal *in vitro* activity decreased to about 1/3 of that of native natamycin. Further, this preparation is comparatively toxic in experimental animals, and it was therefore deemed unsuitable for systemic parenteral administration in humans (15).

SUMMARY OF THE INVENTION

One aspect of the present invention is an antifungal composition that is suitable for parenteral administration to a mammal. The composition includes an amount of pimarin or an antifungal derivative thereof that is effective to inhibit the growth of a systemic infection in a mammal; a pharmaceutically acceptable dipolar aprotic solvent; and a pharmaceutically acceptable aqueous secondary solvent. Suitable dipolar aprotic solvents include N,N-dimethylacetamide (DMA) and dimethyl sulfoxide (DMSO). The aqueous secondary solvent can be, for example, water, saline solution, or dextrose solution. It can also be an aqueous lipid emulsion. Suitable aqueous lipid emulsions include those that comprise a lipid component that includes at least one vegetable oil and at least one fatty acid. In one particular embodiment of the invention, the lipid component comprises at least about 5% by weight soybean oil and at least about 50% by weight fatty acids. The lipids in the composition are preferably present in a form other than liposomes (e.g., at least about 50% by weight of the lipid is not in the form of liposomes, more preferably at least about 75%, and most preferably at least about 95%).

1 Another aspect of the present invention concerns a method of preventing or
2 treating a systemic infection in a mammal. The method comprises administering
3 parenterally to a mammal a composition as described above, in an amount that is
4 effective to inhibit the growth of a systemic infection in the mammal. Although the
5 present invention is especially useful for preventing or treating systemic fungal
6 infections, it can also be used for prevention and treatment of systemic infections caused
7 by other infectious agents that are sensitive to pimaricin in vivo, such as viruses.

8 Another aspect of the present invention concerns a method of preparing an
9 antifungal composition for internal use in a mammal, especially a human. This method
10 includes the steps of dissolving pimaricin or an antifungal derivative thereof in a
11 pharmaceutically acceptable dipolar aprotic solvent; and adding to the solution a
12 pharmaceutically acceptable aqueous secondary solvent. In one preferred embodiment,
13 the method further includes the step of lyophilizing the composition, whereby the
14 majority of the water and the aprotic solvent (e.g., more than 50%, preferably more than
15 95%, and most preferably more than 99% by weight) are removed from the composition
16 and a dry, shelf-stable composition is produced. This dry composition can be
17 reconstituted into an aqueous solution suitable for parenteral administration to a mammal.
18 by adding to the dry composition a pharmaceutically acceptable aqueous solvent.
19 Suitable pharmaceutically acceptable aqueous solvents for reconstituting the composition
20 include the known parenteral infusion fluids, such as saline solution and dextrose solution
21 in addition to distilled water.

22 We have examined the available methods for solubilization and devised nontrivial
23 procedures for solubilizing this agent for parenteral use: we have dissolved it using an
24 organic solvent as the primary vehicle, e.g. dimethylacetamide, and then followed with
25 secondary cosolvents to increase the drug's stable aqueous solubility, or alternatively, we
26 have followed the primary solubilization step with a second aqueous solvent followed by
27 lyophilization to create a pimaricin solvate with minimal organic solvent content, yet one
28 that could be easily reconstituted using distilled water only. Employing a variety of
29 chemical and biological assays we showed that the resulting final pimaricin formulations
30 are stable for several hours at room temperature, and that they retain full antifungal

activity. We ultimately used one of the formulations in a canine model to demonstrate that the reformulated pimaricin permits what has heretofore been impossible, namely safe parenteral (e.g., intravascular) administration with negligible toxicity, yielding clearly fungicidal plasma concentrations for more than six hours following the administration.

The present invention provides vehicles for the formulation of pimaricin that are physiologically compatible with parenteral administration in man and domestic animals. The pimaricin formulations of the present invention are non-toxic and can be used for the parenteral treatment of systemic infections sensitive *in vitro* to this compound, such as infections of *Candida*, *Aspergillus*, and *Fusarium*, to circumvent the virtually nonexistent intestinal absorption of the drug. The invention will allow the introduction of pimaricin in clinical practice for the therapy of systemic infections, such that the therapeutic outcome for patients with systemic infections sensitive to the drug can be improved.

A high-pressure chromatography technique that allows the accurate determination of low concentrations of pimaricin in various solvent systems and in biological fluids. This patent also describes our *in vivo* canine model for studying the pharmacokinetics of pimaricin after parenteral administration.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: Chemical structure of pimaricin as free drug.

Fig. 2: Stability of pimaricin in DMA alone at 4°C (◆), and at RT (22°C) (□), at a concentration of 100 mg/ml. The y-axis shows the fraction of drug remaining as percentage of control (i.e, starting concentration).

Fig. 3: HPLC chromatogram of pimaricin in the HPLC assay. Fig. 3a: Pimaricin extracted from an aqueous solution of 5 µg/ml. Fig. 3b: Pimaricin extracted from a plasma sample spiked to a concentration of 5 µg/ml.

Fig. 4: Stability of pimaricin at 4°C, 22°C, 40°C, and 60°C. The pimaricin formulation was in DMA-aqueous lipid emulsion prepared “fresh.” “AUC” is the area under the curve of the pimaricin peak in the chromatogram. This represents drug concentration, but in this experiment it was not translated into a numerical drug concentration using a standard curve plotting AUC vs. drug concentration.

Fig. 5: Stability over 48 hours of the final solution for clinical use, maintained at RT after dilution to 1 mg/ml. The symbols refer to the following solutions: Pimaricin/L/NACL: the lyophilized and reconstituted solution was diluted from 10 mg/ml to 1 mg/ml with NS. Pimaricin/L/D5: as above, but the secondary solvent was 5% dextrose instead of NS. Pimaricin/NACL: the DMA/Intralipid™ formulation was prepared fresh to a concentration of 10 mg/ml as described, and the secondary solvent used was NS. Pimaricin/D5: The same DMA/Intralipid™ formulation as above, prepared fresh, but the secondary solvent was 5% dextrose instead of NS.

Fig. 6: Hemolytic effects of the DMA/DMSO/PEG/PG formulation without (○) and with pimaricin (●).

Fig. 7: Hemolytic effect of the freshly prepared DMA/aqueous lipid formulation without (□) and with pimaricin (Δ). Negative control was 10% aqueous lipid (Intralipid™) alone (○), at a concentration comparable to that when pimaricin was added to the vehicle at the concentration indicated on the abscissa.

Fig. 8: Hemolytic effect of the DMA/aqueous lipid solution lyophilized and reconstituted in double-distilled water without (■) and with pimaricin (▲). Negative control was the 10% aqueous lipid (Intralipid™) alone (○), at a concentration comparable to that when pimaricin was added to the vehicle at the concentration indicated on the abscissa.

Fig. 9: Pimaricin formulated fresh in DMA/aqueous lipid was assessed for toxicity against the KBM-7/B5 cells (■), and against HL-60 cells (▲), using the MTT assay for 48 hours (Fig. 9a), and for 72 hours (Fig. 9b) as described in materials and methods.

Fig. 10: HPLC chromatograms of a plasma sample analyzed with the HPLC assay. Fig. 10a: Plasma blank samples before the start of infusion. Fig. 10b: Sample from a dog injected with 5 mg/kg body weight of pimaricin. The drug was given over 1 hour iv and this blood sample was obtained 5 hours after drug infusion was completed. The sample was extracted and analyzed as described in the text.

Fig. 11: Dose linearity of pimaricin utilizing the established HPLC assay in the concentration range 100 ng/ml to 25 µg/ml.

Fig. 12: Comparative plasma concentrations during and after infusion of pimarinic at 1 mg/kg, and 5 mg/kg in four beagle dogs. The samples were drawn just before the end of the 60 min infusion and 5 hours after the end of infusion. The different numbers and symbols, respectively, refer to the individual animals, and the 1 and 5 respectively refer to the dose of pimarinic administered per kg body weight.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The following abbreviations are used in this patent:

AMB; Amphotericin B.

ATCC; American Tissue Culture Collection, Rockville, MD.

BMT; bone marrow transplant.

DMA; anhydrous N,N,-dimethylacetamide.

DMF; Dimethylformamide.

DMSO; Dimethylsulfoxide.

FDA; U.S. Food and Drug Administration.

HAc; Glacial acetic acid.

HCl; Hydrochloric acid.

HPLC; High pressure liquid chromatography.

HL-60; Human myeloid leukemia cell line.

IMDM; Iscove's modified Dulbecco Medium (GIBCO, Grand Island, New York, NY).

Intralipid™; Brand name of an aqueous lipid emulsion, made from soy bean oil, and marketed for parenteral nutrition by Clintec.

KBM-7/B5; Human myeloid leukemia cell line.

MeOH; Methanol.

MIC; minimum inhibitory concentration.

MTT; 3,[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium-bromide.

NCI; National Cancer Institute.

NS; Normal saline (150 mM NaCl).

PEG; Polyethylene glycol-400.

PG; Polypropylene glycol/1,2-propylene diol.

1 RT; Room temperature (22°C)

2 SDS; Sodium dodecyl sulphate.

3
4 The present invention involves solubilization of pimaricin in pharmaceutically
5 acceptable liquid vehicles, such that the drug remains chemically stable and can be
6 administered intravascularly without undue toxicity from undissolved drug and/or from
7 the solvents at drug doses necessary to obtain clinically significant antibiotic effects.

8 Pimaricin is available from Gist-Brocades N.V. (Netherlands) and Sigma
9 Chemical Co. (Saint Louis, Missouri). Pimaricin optionally can be used in compositions
10 of the present invention in the form of one of its antifungal derivatives, such as a salt of
11 pimaricin (e.g., an alkali salt or an alkaline earth salt).

12 We have investigated N,N-dimethylacetamide (DMA), DMSO, glycerol, 1,2,-
13 propylene-diol (PG), and polyethylene glycol-400 (PEG) as primary solvents that would
14 be miscible in secondary solvents, examples of which are normal saline, dextrose in water
15 (5% or 10%), and an aqueous soy bean lipid emulsion (Intralipid™). These solvents are
16 examples of vehicles in which pimaricin could be suitably solubilized, yet be safe for
17 human administration, alone or in combinations with other drugs. The solubility of
18 pimaricin in individual solvent vehicles is shown in Table 1 below.

19 The described vehicles can be utilized to dissolve pimaricin in concentrations
20 ranging from 1 to more than 100 mg/ml. This range should cover the administration of
21 doses necessary to yield active antibiotic concentrations *in vivo* that are effective to
22 eradicate systemic infections sensitive to this drug.

23 The objective of this invention includes the parenteral (e.g., intravascular)
24 administration of pimaricin to improve the control of systemic infections that are
25 sensitive to this agent. As a paradigm for such infections, we will use various molds and
26 other fungal organisms. This use of pimaricin as a parenteral agent has not been
27 previously investigated in the practice of medicine, although the drug has well
28 documented anti-fungal properties *in vitro* (15-17).

29 Virtually no pimaricin is absorbed through the intestinal tract after oral
30 administration, making it impossible to even investigate its use as an oral antibiotic

1 against systemic infections. Parenteral administration would therefore be the logical
2 approach to evaluate pimaricin as therapy for deep-seated, systemic fungal infections.
3 Unfortunately, the drug has an exceedingly low solubility in most physiologically
4 acceptable solvents that would be compatible with intravascular administration in man
5 (17).

6 Our present studies, which are based on the principle of cosolvency (20, 21), show
7 that the composite diluent vehicles we propose for use will solubilize pimaricin without
8 destroying its antifungal properties. Further, the preferred vehicles are nontoxic and safe
9 for administration in large animals (beagles) and should be acceptable for human
10 administration in the proposed concentrations and total doses to be utilized; indeed,
11 DMA, DMSO, and PG have been used for solubilization of various pharmacologically
12 active agents used in man (22-24). The parenteral administration of PEG has been studied
13 in detail in a simian model (25), and PEG has subsequently been used clinically as a
14 (covalently bound) carrier of L-Asparaginase in the treatment of lymphocytic leukemia
15 and lymphoma (26). DMSO is also extensively used as a cryoprotective agent for low-
16 temperature storage of human bone marrow and peripheral blood derived hematopoietic
17 stem cell preparations to be used for transplantation after high-dose chemotherapy (27-
18 30). No serious adverse effects have been experienced from the use of these vehicles.
19 The clinical use of normal saline, dextrose in water (5-70%), and aqueous lipid emulsion
20 are well established means to alter the fluid and electrolyte balance and to supply
21 parenteral nutrition. Normal saline and dextrose in water are extensively used to dilute
22 various medications for parenteral use. However, the aqueous lipid emulsion has not yet
23 found wide-spread use as a pharmaceutical diluent, although this use has been mentioned
24 (31).

25 The data obtained in our canine model demonstrate that the proposed pimaricin
26 formulations, that is, those that allow parenteral treatment of systemic infections, will
27 provide superior bioavailability. After a one-hour i.v. infusion the plasma concentrations
28 clearly reach, and for an extended time remain in, the fungicidal range as established by
29 our *in vitro* studies of antifungal activity against *Candida* spp., *Aspergillus* spp., and
30 *Fusarium* spp. Specifically, our novel pimaricin/DMA/lipid solution is chemically stable

1 and simple to handle at RT. It provides reliable and easily controlled dosing with 100%
2 bioavailability. The addition of a lyophilization step virtually eliminates the organic
3 solvent, DMA, from the final clinical “working solution”, and it should abolish the
4 potential for adverse reactions related to the DMA, and minimize the possibility for a
5 potentiation of (hepatic) side effects from the combination of DMA and pimarin. This
6 added step should therefore assist in maximizing patient safety after drug administration.

7 In cancer patients, the access to parenteral pimarin will be particularly
8 important, since their intestinal absorption is often perturbed after chemotherapy,
9 aggravating the already erratic intestinal absorption of various medications. The
10 parenteral route will also make it possible to circumvent unpredictable first-pass
11 metabolic effects in the liver, well known to alter the bioavailability of numerous
12 pharmacologically active agents after oral dosing (32). Further, the availability of
13 pimarin for effective and reliable systemic administration will for the first time make it
14 possible to clinically compare the activity of pimarin against that of “the gold
15 standard”, AMB, for the treatment of systemic mycoses.

16 In summary, certain infections in immunocompromised patients, e.g. those caused
17 by various molds, particularly Fusarium, may be eradicated by pimarin. In fact,
18 pimarin may be the only effective drug for the treatment of Fusariosis, since this
19 infection typically is resistant to AMB. The design of a nontoxic, pharmaceutically
20 acceptable, water miscible, parenteral formulation of pimarin eliminates the risk of
21 treatment failure from the suboptimal bioavailability of oral pimarin. The addition of a
22 lyophilization step in the preparative procedure will create a pimarin solvate with
23 minimal DMA content. This will reduce the risk of adverse effects related to the
24 vehicle’s organic component.

25 The following examples are presented to describe the preferred embodiments and
26 utilities of the present invention, but they are not intended to limit the invention to these
27 aspects, unless otherwise stated in the claims appended hereto.

28 **EXAMPLE 1. Pimarin Formulations Acceptable for Parenteral**
29 **Administration.**

1 The objectives of this experiment were to design formulations of pimaricin that
2 are acceptable for parenteral administration, to calculate the necessary solubility/stability
3 needed to accomplish this goal, and to evaluate our ability to make such preparations with
4 a high pressure liquid chromatographic (HPLC) technique.

5 **METHODOLOGY.**

6 **Calculation of the Desired Solubility.**

7 We have calculated a relevant solubility range for pimaricin by extrapolation from
8 known values for AMB. AMB is presently the only polyene antibiotic that is FDA-
9 approved for parenteral use. The currently utilized AMB regimens typically prescribe a
10 daily dose of 0.6-1.0 mg/kg body weight as free AMB or 5-6 mg/kg body weight for
11 liposomally-complexed drug (11). We have assumed that a clinically safe maximum
12 infusion rate for pimaricin is 2-3 ml/min over 60-120 minutes, thus arriving at peak
13 plasma concentrations in the range of 3-15 µg/ml (4.5-20 µM). Such concentrations may
14 be necessary if pimaricin treatment is to be successful, since AMB and pimaricin on a
15 molar basis have a similar concentration vs. activity range *in vitro* (AMB about 0.3-10
16 µM, and Pimaricin about 3-20 µM). Therefore, the anticipated daily pimaricin dose
17 would be around 1.0 - 5.0 mg/kg body weight. If this dose were dissolved at a
18 concentration of 1-5 mg/ml, a 50-100-fold increase over the established aqueous
19 solubility of 25-50 µg/ml at RT would be required (17).

20 **Enhanced Solubility in Physiologically Acceptable Solvents.**

21 Pimaricin solubility was determined in several individual vehicles. Briefly, a
22 known amount of the drug, as a powder (different lots of purified drug were obtained
23 from Gist-Brocades N.V., Netherlands, and from Sigma Chemical Company, St. Louis,
24 MO), was equilibrated in the respective solvent at RT (22°C) over 1-4 hours. An aliquot
25 was then removed and diluted in MeOH prior to HPLC at predetermined times. Based on
26 the pimaricin solubility in these particular vehicles, we then attempted to enhance the
27 (stable) solubility by mixing different solvents according to the principle of cosolvency
28 (20, 21). Several different solvent systems were evaluated relative to the above estimates
29 of necessary solubility to arrive at a clinically relevant optimal stock formulation. This
30 stock formula would then be diluted with a "final solvent" to yield the complete working

1 formulation with a pimaricin concentration that could be infused parenterally without
2 problem. For the final solvent we used the commonly utilized parenteral infusion fluids,
3 such as normal saline, dextrose in water (5% or 10%), or a parenterally acceptable
4 aqueous lipid emulsion (e.g. Intralipid™ or Liposyn II™ (Abbott)), all of which are
5 readily available and approved for parenteral administration.

6 **HPLC Assay.**

7 A most accurate and sensitive detection system for low concentrations of
8 pimaricin in solution, both protein-containing and protein-free mixtures, is an HPLC
9 assay utilizing absorbance detection with a variable wave length detector operating in the
10 u.v. spectrum at 293 nm, a value chosen on the basis of the inherent absorption maxima
11 of the pimaricin molecule (17).

12 We tested this hypothesis using a liquid chromatographic system equipped with
13 an LDC 4000™ multi-solvent delivery system and a Waters™ system 717*plus*
14 Autoinjector™. The absorbance detector was a LDC 3100 variable wave length detector
15 in sequence with an LDC model CI 4100 fully computerized integrator. The column used
16 was a Whatman EQC™ 10 µI 125A C18 column (4.6 mm i.d. x 21.6 cm) (Whatman Inc.
17 Clifton, NJ). The mobile phase system was an isocratic mixture of MeOH (47% v/v),
18 tetrahydrofuran (2% v/v), and NH₄-acetate (0.1% w/v) made up to 100% with double-
19 distilled water. All chemicals were HPLC grade unless otherwise indicated. The flow
20 rate was 1.5 ml/min and the recorder's chart speed was 5 mm/min, modified from (33).

21 **RESULTS AND DISCUSSION.**

22 **Pimaricin Solubility.**

23 Several strategies were evaluated to solubilize pimaricin in water-miscible
24 physiologically acceptable vehicles that would be compatible with human administration.
25 The examined candidate solvents included castor oil, DMA, DMSO, PEG, and PG, in
26 addition to the aqueous solvents HAc, NS, 5% dextrose in water and an aqueous soy bean
27 emulsion (Intralipid™). HAc and DMA were the best primary solvents, followed by
28 DMSO, whereas pimaricin as expected was insoluble in most of the aqueous solvents.
29 Only with HAc and DMA did we reach a solubility in excess of 10 mg/ml. Further,
30 although pimaricin could be dissolved in HAc and DMA to at least 100 mg/ml, it started

degrading already within a few hours in solution (Fig. 2). Stabilizing the pimaricin once dissolved in DMA was then addressed with a cosolvency approach (20, 21). Numerous cosolvent combinations were investigated; the composite organic system of DMA/DMSO/PEG/PG appeared to work well, but it did still only allow pimaricin to be dissolved at a final concentration of about 10 mg/ml. This composite vehicle did not allow stable solubilization of pimaricin for more than a few hours. When NS or 5% dextrose in water was added, significant degradation rapidly took place. In contrast, a different pattern was recorded when a lipid-containing cosolvent was utilized. When HAc was used as the primary solvent, the best secondary solvents appeared to be DMA, DMSO or Intralipid™.

HPLC Assay.

Two examples of pimaricin chromatograms from the HPLC assay are shown in Figure 3. In Fig. 3a the drug was analyzed in the aqueous DMA-Intralipid solvent, and in Fig. 3b it was extracted from human plasma that had been spiked with 5 µg/ml prior to extraction as described above. The retention time under the above conditions was 9.8-10.8 min, and the assay was linear from 100 ng/ml to 25 µg/ml in protein-free solutions, i.e. the various solvent systems utilized in the formulation-feasibility and -stability studies, and from about 50 ng/ml to 1 mg/ml for protein-containing solutions (plasma samples). This assay consistently yielded high recovery, accuracy and a lower sensitivity limit of about 10 ng/ml. The technique was standardized and used without modifications for the studies of both stability and pharmacokinetics.

EXAMPLE 2. Solubility and Stability Studies of Various Formulations.

The objectives of this experiment were to: (1) design stable pimaricin formulations that are suitable for parenteral administration; (2) establish the chemical and physical stability of pimaricin in the novel vehicles; (3) establish the solubility of pimaricin in these vehicles when mixed with NS, dextrose in water, and Intralipid™; and (4) investigate the *in vitro* properties of these formulations: i.e. their osmolarity, hemolytic potential, and cytotoxicity, to show that they are appropriate for the intended purpose.

METHODOLOGY.

Solubility Studies.

An excess amount of pimaricin as a solid powder was added to castor oil, DMA, DMSO, PEG, and PG at RT. Each mixture was placed in a dark environment and checked visually for up to 4 hours for evidence of solubilization. Samples of 1 ml were taken at various time intervals, and filtered through a 0.45 μ m PTFE membrane filter fitted to a syringe assembly (Whatman Inc.), and after appropriate dilution, the pimaricin concentration was determined by HPLC.

Stability of the Various Pimaricin Formulations.

To study the physical and chemical stability of the various parenteral formulations, three sets of experiments were performed:

(a) Pimaricin was dissolved at a concentration of 100 mg/ml in DMA ("stock solution") and incubated at 4°C, at 22°C and at 40°C. We analyzed the drug concentration by HPLC in samples taken immediately after solubilization and after gradually increasing time intervals of up to 48 hours.

(b) The pimaricin-DMA stock solution was diluted with PEG/water (1:1:1, v:v:v, DMA:PEG:water), or PG/DMSO (1:1:1, v:v:v), or PG/DMSO/PEG (1:1:1:1, v:v:v:v), or aqueous lipid emulsion (1:10 and 1:100, v:v, DMA:Intralipid™), to yield pimaricin concentrations ranging from 1-10 mg/ml.

(c) The DMA-pimaricin mixture was diluted in NS or 5% dextrose to a drug concentration of 1 mg/ml.

(d) The pimaricin-HAc mixture was blended with DMSO and Intralipid™, or directly in Intralipid™.

The various formulations were analyzed by HPLC immediately after mixing, then hourly for 8 hours, and then at gradually increasing time intervals up to several weeks, depending on the rate of degradation in the respective solvent system.

The solubility of the drug differed markedly between different solvents (Table 1). Only DMA and HAc, which provided the highest solubility were considered for extended studies as primary solvents.

Table 1

Solvents Tested for Solubilization of Pimaricin

| Formulation | Time Allowed to Solubilize (hr) | Maximum Solubility (mg/ml) | Vehicle |
|-------------|---------------------------------|----------------------------|------------|
| 1 | 4 | 2 | DMSO |
| 2 | 4 | 10 | DMA |
| 3 | 6 | 100 | DMA |
| 4 | 4 | 0.078 | PG |
| 5 | <0.2 | >300 | HAc |
| 6 | 4 | N/S | Castor oil |
| 7 | 4 | N/S | PEG400 |
| 8 | 4 | N/S | Intralipid |

(N/S indicates that pimaricin was not soluble in that solvent.)

To lower the DMA concentration in the final stock- and use-formulations without adversely affecting the drug's shelf life, we investigated lyophilization as part of the preparation of a complete pimaricin/DMA/aqueous lipid-solvate vehicle.

Osmotic Pressure Measurement.

Osmotic pressures were measured with a micro-osmometer model 3MOplus osmometer (Advanced Instruments Inc., Needham Heights, MA). The instrument was calibrated using Advans™ intrinsic calibration standards (Advanced Instruments Inc.) over a range of 500-2000 mOsm/kg. The test solution was placed in a disposable cuvette from the test kit, and the osmotic pressure readings were recorded after equilibration in units of mOsm/kg. Triplicate measurements were carried out for each vehicle (without pimaricin), and six measurements were done with pimaricin added.

We used a two-tailed t-test to evaluate the differences in osmotic pressures of the various vehicle formulations with and without the addition of pimaricin (34). The difference between the means of the two groups was to be considered significantly different for $P \leq 0.05$.

Hemolysis Studies *in vitro*.

We employed the procedure of Parthasarathy et al to examine the hemolytic potential of a few selected preparations (35), and the LD₅₀ values of the various formulations were constructed as described. Briefly, heparinized blood was mixed with an equal volume of Alsever's solution. This mixture was washed twice in PBS, and a 10% (v/v) erythrocyte/PBS solution was then prepared and mixed with increasing amounts of the complete solvent system with or without the addition of pimaricin. These mixtures were then incubated for 4 hours at 37°C. At the end of the incubation, the cells were pelleted at 10,000 x g in an Eppendorff™ centrifuge, and the release of hemoglobin in the supernatant (i.e. hemolysis) was spectrophotometrically determined at 550 nm. Maximum lysis was measured against a reference solution of erythrocytes that had been completely lysed by hypotonic shock. The hemolytic potential of three of the complete formulations was evaluated as described (35), and the data were plotted as the fraction of healthy cells versus ln (natural logarithm) (total volume percent). Total volume percent was defined as the volume percent of the vehicle in the mixture after dilution with blood. This was done in an attempt to simulate the dilution of the respective drug formulation in the bloodstream after parenteral administration. Healthy erythrocytes were defined as those capable of retaining their hemoglobin intracellularly after mixture with the various pimaricin formulations (35).

In Vitro Cytotoxicity of Pimaricin.

The cytotoxic potential of selected solvent systems with and without pimaricin was determined against the two human myeloid leukemia cell lines HL-60 (36) and KBM-7/B5 (37, 38), using a modification of the previously published MTT assay (39, 40). Briefly, HL-60 or KBM-7/B5 cells in Iscove's modified Dulbecco medium (IMDM) supplemented with 10% fetal bovine serum were incubated for 60 min at 37°C with the complete vehicles (a: DMA/PG/DMSO/PEG in ratios 1:1:1:1, v/v, and b: DMA/Intralipid™, 1:10, v/v, or c: HAc/DMSO/Intralipid™, 2:6:3, v/v) at increasing concentrations of the vehicle (0.5%, 1.0%, 2.0%, 3.0%, and 10%, v/v) with or without pimaricin. At the end of the 60 min incubation the cells were washed in ice-cold PBS and resuspended in IMDM with 10% fetal bovine serum at 37°C. Twenty-four hours later 25 µl MTT solution (5 mg/ml) (Sigma Chemicals, St. Louis, MO) was added to each

sample, and following an additional 2 hours of incubation at 37°C, 100 µl extraction buffer was added [extraction buffer: 20% (w/v) SDS dissolved to saturation at 37°C in a solution of DMF and deionized water (1:1); pH 4.7]. After incubation overnight at 37°C, the optical densities were measured at 570 nm using a Titer-Tech™ 96-well multi-scanner™, against extraction buffer as the calibrating blank. The cytotoxicity was determined as the colorimetric difference between the samples exposed to solvent ±pimaricin as above and the background reactivity of cells that had been incubated in parallel in PBS alone. All determinations were performed in triplicate (39, 40).

RESULTS AND DISCUSSION.

Equilibrium Solubility Determinations and Stability Studies in Various Solvent Vehicles.

A maximum equilibrium solubility of pimaricin of >100 mg/ml was achieved in DMA after 4 hours at RT. The drug formulations in castor oil, DMSO, PEG-400 and PG achieved considerably lower equilibrium concentrations (Table 1). The latter solvents neither provided an acceptable solubility nor chemical stability of the dissolved drug, and these vehicles were therefore not considered for further studies. Once a pimaricin solubility of 100 mg/ml was reached in anhydrous DMA and HAc respectively, the drug started degrading with a loss of approximately 5-10% over the subsequent 3-4 hours. The drug was more stable when PEG was used as a secondary solvent, but again drug degradation began within another few hours at RT. At 4°C the drug was more stable, but degradation was still apparent within 8 to 12 hours.

The temperature-dependent stability of solubilized pimaricin in the different solvent systems was studied as follows: The drug was dissolved in DMA at 100 mg/ml. and different aliquots were stored at 4°C. at 22°C. and at 40°C. Immediately after solubilization and at various intervals up to 48 hrs later, aliquots from the different samples were analyzed by HPLC. The drug samples stored at 4°C and at 22°C degraded slower than those stored at higher temperatures: at 40°C the pimaricin started degrading within 1 hour after the start of incubation. and at RT there was a loss of 5-10% in the first four hours.

1 When the 20% aqueous lipid emulsion (Intralipid™) was used as a secondary
2 solvent, a different stability pattern was recorded; when the pimarin concentration was
3 adjusted to 1-10 mg/ml by dilution with 20% Intralipid of the DMA-pimarin and the
4 HAc-pimarin stock solutions, the drug was stable for more than 7 days (Fig. 4).

5 The major fraction of the organic solvent, DMA, was removed by lyophilization
6 of the pimarin/DMA/aqueous lipid complex to create a solvate that was stable yet easily
7 reconstituted by adding only double-distilled water under gentle agitation without any
8 appreciable loss of anti-fungal efficacy. Indeed, within a few minutes after addition of
9 distilled water to the solvate, the drug was reconstituted at 1-10 mg/ml, with only trace
10 amounts of the organic solvent remaining. This reconstituted pimarin formulation
11 retained an anti-fungal efficacy that was equivalent to that of the freshly prepared
12 DMA/aqueous lipid formulation when assayed *in vitro* (see below under Example 3).
13 This reconstituted formulation was also stable at 4°C for more than 2 weeks. The
14 lyophilized pimarin formulation remained stable (by HPLC) for more than four months
15 at 4°C. This preparation could still be readily reconstituted to 10 mg/ml within a few
16 minutes with distilled water, with retention of full anti-fungal activity *in vitro* (see Tables
17 3 and 4 below).

18 We further simulated a final clinical use-formulation with a pimarin solution of
19 1 mg/ml by diluting the 10 mg/ml-formulations (prepared fresh with DMA/Intralipid or
20 after lyophilization/reconstitution respectively) with 5% dextrose or NS. Figure 5 shows
21 the respective stability at RT of these "use-formulations". Similarly, when HAc and
22 DMSO were used as the primary solvent system prior to mixing with Intralipid and
23 followed by lyophilization, the majority of the organic solvent, here DMSO, was
24 removed and the result was a stable lipid-based solvate, that could be easily reconstituted
25 to 10 mg/ml under gentle agitation after the addition of distilled water. This reconstituted
26 formulation was also stable for more than 24 hours at RT assessed by HPLC.

27 **Osmotic Pressure.**

28 It is desirable that a parenteral formulation of a pharmacologically active agent be
29 isosmotic to blood. A hypertonic delivery system can be utilized if the drug/solvent is

infused through a (central) venous catheter and gradually diluted in a large blood volume. The osmotic pressure of the various formulations is shown in Table 2.

Table 2
Osmotic Pressures of Various Vehicles with and without Pimaricin

| Solution | n | Osmotic pressure mOsm/kg |
|---|---|-----------------------------|
| Water | 3 | 3 |
| Normal saline | 3 | 233 |
| 5% dextrose in water | 3 | 286 |
| Blood, human | 6 | 280-295 |
| DMA:PEG:PG | 3 | 4492 |
| Pimaricin in DMA:PEG:PG | 3 | 4732 |
| Intralipid | 3 | 340 |
| DMA:Intralipid (1:10, v/v) | 3 | 2067 |
| Pimaricin in DMA:Intralipid (1:10, v/v, fresh) | 3 | 1930 |
| DMA:Intralipid (1:10, lyophil.-reconstit.) | 3 | 157 |
| Pimaricin (1 mg/ml) in DMA:Intralipid (1:10, lyophil.-reconstit.) | 3 | 208 |
| Pimaricin (25 mg/ml) in DMA:Intralipid (1:10, lyophil.-reconstit.) | 3 | 243 |

("n" represents the number of independent determinations.)

The DMA-stock formulation with or without pimaricin was very hypertonic; its osmotic pressure was more than 1,900 mOsm/kg, as compared with 280-295 mOsm/Kg for human blood. The DMA/PG/DMSO/PEG and DMA/PEG solvents were almost as hypertonic. In contrast, the DMA/Intralipid preparation was closer to isosmotic when reconstituted after lyophilization. Similarly, the lyophilized/reconstituted HAc/DMSO/Intralipid™ vehicle was also close to isosmotic. Adding pimaricin to the respective vehicles did not appreciably change their osmolarity ($P > 0.05$).

Hemolysis.

As shown in Figures 6-8, the formulations studied showed similar trends for hemolysis with the addition of pimaricin. The pimaricin dependent lysis was notable at concentrations exceeding 40 µg/ml for the composite organic solvent and at ≥50 µg/ml for the freshly prepared DMA/Intralipid formulation and at ≥60 µg/ml for the lyophilized-reconstituted DMA/aqueous lipid formulation. The drug-specific hemolysis

was highly reproducible between different experiments, as was the internal ranking between the various solvent systems between the different experiments. The detailed data for the different vehicles with and without pimaricin are summarized in Figures 6-8. LD₅₀ values can be deduced from this information. The DMA/Intralipid™ “fresh” formulation had a significantly lower hemolytic potential than the DMA/PEG/PG/DMSO composite organic vehicle. Further, the hemolytic potential of the lyophilized DMA/Intralipid formulation was significantly lower than that of the freshly prepared DMA/aqueous lipid formulation for all pimaricin concentrations from 1 µg/ml up to 100 µg/ml. Finally, pimaricin-induced hemolysis in all of the tested vehicles was significantly lower (>10-fold) than that observed for various AMB formulations (LD₅₀ values in the range of about 4-5 µg/ml) under similar experimental conditions (41).

In Vitro Cytotoxicity of Pimaricin.

The HL-60 and KBM-7/B5 myeloid cells were exposed to the selected vehicles at increasing volume ratios with or without the addition of increasing drug concentrations. The cytotoxicity of each formulation was then assayed in the MTT assay (39, 40). None of the examined solvent systems exerted any detectable toxicity against the cells in this assay (Fig. 9).

EXAMPLE 3. Antifungal Activity of Solubilized Pimaricin.

The objective of this experiment was to critically evaluate the in vitro antifungal activity of pimaricin when solubilized in a few selected vehicles using solution AMB as the reference solution.

METHODOLOGY.

The antifungal activity of pimaricin was compared with that of amphotericin B utilizing a previously described assay (42). Briefly, serial dilutions of pimaricin and AMB were mixed in RPMI growth medium with L-glutamine and MOPS-buffer, pH 7.0 (Sigma Chemical Co., St. Louis, MO). The different strains of Candida, Aspergillus and Fusarium spp. were then added to the dishes. After incubation at 35°C for 48-72 hours the plates were evaluated for fungal proliferation. The used fungal strains were obtained from the ATCC or isolated from patients, primarily at the MD Anderson Cancer Center.

The pimarin concentrations in the used solutions were assayed in parallel with HPLC to assure the highest possible reproducibility of the drug concentrations.

RESULTS AND DISCUSSION.

The sensitivity data are displayed in Tables 3 and 4.

Table 3

Sensitivity of Fungal Organisms Against Various Pimaricin Formulations

| Organism | Code | L/D µg/ml | Rm-temp µg/ml | F/D (nata+lipid) µg/ml |
|-----------------------|------------|--------------|------------------|------------------------------|
| Aspergillus fumigatus | 6-2535 | 2 | 2 | 2 |
| Aspergillus fumigatus | 6-7784 | 2 | 2 | 2 |
| Aspergillus niger | 6-2165 | 2 | 2 | 2 |
| Aspergillus fumigatus | 6-5337-1 | 2 | 2 | 2 |
| Fusarium moniliformi | M6306 | 2 | 2 | 2 |
| Aspergillus flavus | 6-4594-2 | >16 | >16 | >16 |
| Fusarium solanii | s-1184 | 2 | 2 | 2 |
| Candida albicans | ATCC 64545 | 2 | 2 | 2 |

The organisms of Table 3 were prepared as specified in the methodology in Example 3. "L/D" refers to a formulation where pimarin was dissolved to 100 mg/ml in DMA, then diluted to 10 mg/ml with 20% Intralipid, lyophilized and then stored for >4 months at 4°C. followed by reconstitution in normal saline to 10 µg/ml as "use-solution". "Rm-temp" refers to a formulation where pimarin was prepared fresh in DMA and Intralipid (10 mg/ml), kept for one week at RT. and then tested for its antifungal properties. "F/D (Nata+lipid)" refers to a formulation where pimarin was freshly dissolved at 100 mg/ml in DMA and then diluted with 20% Intralipid to 10 mg/ml as a fresh use-solution that was diluted to final concentrations of <2 to 16 µg/ml as described herein.

Table 4 reports the results of another similar experiment.

Table 4

Sensitivity of Fungal Organisms Against Various Pimaricin Formulations

| Organism | Code | Lipid+DMA (1:10) µg/ml | Nata-lipid 1 µg/ml | Nata-lipid 2 µg/ml | AMP+DMSO µg/ml |
|------------------------------|----------|------------------------------|-----------------------|-----------------------|-------------------|
| <i>Aspergillus fumigatus</i> | 6-2535 | >16 | 2 | 2 | 0.125 |
| <i>Aspergillus fumigatus</i> | 6-7784 | >16 | 2 | 2 | 0.25 |
| <i>Aspergillus niger</i> | 6-2165 | >16 | 2 | 2 | 0.03 |
| <i>Aspergillus fumigatus</i> | 6-5337-1 | >16 | 4 | 4 | 0.5 |
| <i>Aspergillus flavus</i> | 6-4594-2 | >16 | >16 | >16 | 1 |
| <i>Aspergillus fumigatus</i> | 6-209 | >16 | 2 | 2 | 0.25 |
| <i>Aspergillus fumigatus</i> | 6-0960 | >16 | 2 | 2 | 0.25 |
| <i>Aspergillus fumigatus</i> | 6-1886 | >16 | 4 | 4 | 0.25 |
| <i>Aspergillus fumigatus</i> | 6-1261 | >16 | 4 | 4 | 0.25 |
| <i>Aspergillus flavus</i> | 4-9044 | >16 | >16 | >16 | 1 |
| <i>Aspergillus flavus</i> | 6-5337-2 | >16 | >16 | >16 | 1 |

“Lipid+DMA” refers to freshly mixed DMA and Intralipid (1:10, v/v), which exerts no antifungal activity by itself. For “Nata-lipid 1” and “Nata-lipid 2,” pimaricin was dissolved in DMA to 100 mg/ml then diluted with 20% Intralipid to 10 mg/ml “use-formulation.” “Nata-lipid 1” refers to a formulation where pimaricin was dissolved as above, and after dilution to 10 mg/ml using Intralipid, it was lyophilized. The lyophilized material was refrigerated for 4 months, then reconstituted in normal saline to 10 mg/ml and tested for antifungal activity. “Nata-lipid 2” refers to a formulation where the pimaricin/DMA/Intralipid formulation was prepared as for Nata-lipid 1 and lyophilized immediately, and was reconstituted and tested for antifungal activity three days later. “AMP+DMSO” refers to a formulation of Amphotericin B dissolved immediately prior to use in DMSO, to serve as a positive control.

The activity of pimaricin was similar to that of AMB. Most of the *Aspergillus* and *Fusarium* spp. were sensitive to pimaricin, independent of the solvent system. Importantly, the DMA/Intralipid™ formulation that was lyophilized and reconstituted with distilled water only, retained full and stable anti-fungal efficacy, when assayed both after 3 days and after more than 4 months at 4°C. All the *Aspergillus* strains, except for *A. flavus*, had pimaricin MIC values in the 2-4 µg/ml (2.1-4.2 µM) range. The tested *A. flavus* was also sensitive to the drug, but with a slightly higher MIC value of 16 µg/ml

(17 μ M). All the tested strains of *Fusarium* and *Candida* spp. were sensitive to pimarinic in the range of 2-4 μ g/ml (Tables 2 and 3).

EXAMPLE 4. Quantitative Pimaricin Analysis in Plasma and Pharmacokinetics of iv Pimaricin.

The objective of this experiment were:

(1) To show that the drug can be administered intravenously and recovered from the plasma from experimental animals using a quantitative extraction technique and HPLC assay; and

(2) To show that the pimarinic plasma pharmacokinetics after iv administration of the DMA/20% aqueous lipid formulation in beagle dogs are appropriate for treating systemic microbial diseases, in particular Fusariosis.

Methodology.

Quantitative Extraction of Pimaricin in Plasma.

Canine plasma (0.2 ml) and human plasma (0.5 ml) were mixed with various amounts of pimarinic (in <3% of the final volume), to yield a drug concentration of 0.05-3.0 μ g/ml (from a pimarinic stock solution in DMA/20% Intralipid™ at a concentration of 10 mg/ml). The drug was extracted from plasma samples using a slight modification of the method described by Napoli et al (43). Briefly, 0.2 ml plasma was mixed with 0.2 N HCl in MeOH (1:1, v/v), and after thorough mixing by a vortex machine, the sample was extracted with three volumes of hexane. The hexane was separated from the pimarinic by evaporation and the drug was reconstituted in 200 μ l of MeOH prior to HPLC (43). Pimaricin was spectrophotometrically detected in the HPLC analysis as described above on page 14. The pimarinic recovery from human plasma spiked to a pimarinic concentration of 10 μ g/ml was calculated to be 91 \pm 5%, and from canine plasma it was estimated to be in the order of 85 \pm 4%. The assay was linear in the interval from 50 ng/ml to at least 1,000 μ g/ml.

Parenteral Pimaricin in Beagles: Experimental Protocol.

For the pharmacokinetics experiment we elected to use beagle dogs, since these animals are exceedingly sensitive to the toxic adverse effects of polyene antibiotics, and particularly to the nephrotoxic effects of these agents. The pimarinic was formulated in

1 DMA/Intralipid™ to a stock drug concentration of 10 mg/ml, and then diluted with
2 Intralipid™, so the doses (1.0 mg/kg/day in two dogs and 5.0 mg/kg/day in two other
3 dogs) could be administered IV in a volume of 10 ml over 1 hour by pump through a
4 cephalic vein catheter. To assure reproducibility of the experimental conditions, the
5 infusions were staggered; one dog at each dose level was started on two consecutive days.
6 The investigation was performed in male beagle dogs weighing 10-14 kg. The animals
7 were not anesthetized but were restrained in a hanging sling during the drug infusion,
8 which was performed at the same time daily for 14 consecutive days. EKGs were
9 recorded and blood samples were obtained for determination of pimaricin concentrations
10 prior to the drug infusion and at various times during and following the infusion on the
11 first day and on the last day of drug infusion. Blood for analysis of liver and kidney
12 function, as well as for differential and complete blood counts, and platelet counts, was
13 obtained in the morning before the first drug infusion, and also on days 8 and 15.

14 All animals were allowed free access to food and water, but with some restriction
15 to space and mobility, since we were concerned that parenterally administered pimaricin
16 could be cardiotoxic and cause fatal arrhythmias in a fashion similar to that of AMB,
17 another polyene antibiotic.

18 The drug was administered through the cephalic vein with good tolerance. The
19 cannula and tubing were carefully flushed with heparinized saline after each injection to
20 prevent clot formation and to prevent drug from adhering to the catheter wall and thus
21 interfering with the blood sampling for routine chemistries and for the pharmacokinetic
22 analysis.

23 Blood samples of 3 ml were drawn in heparinized tubes before drug infusion, and
24 at 10, 30, 55, 65, 70, 80, and 100 min, and at 2, 4, 6, 12, 18, and 24 hours after the start of
25 the infusion. The blood was centrifuged at 1,000 x g for 10 min, and the plasma was
26 separated and stored at -80°C until assayed by HPLC.

27 **RESULTS AND DISCUSSION OF THE DATA.**

28 **Pimaricin in Plasma and iv Drug Pharmacology.**

29 The drug extraction with hexane and MeOH from plasma was essential to avoid
30 interference from endogenous plasma components and to recover the maximum amount

of drug. Chromatograms from blank plasma, pimarinin-spiked plasma, and one example of that obtained after extraction of a plasma sample from the current pharmacokinetic study are shown in Fig. 10. The pimarinin retention time in this system was 9.8-10.8 min. The recovery of pimarinin with the above described technique was $91 \pm 5\%$ when human plasma was spiked *in vitro* with 10 $\mu\text{g/ml}$ of drug. The assay was linear after drug extraction from plasma samples in the range from 50 ng/ml to 1.0 mg/ml. The drug recovery from canine plasma was $85 \pm 4\%$, with an accuracy of 98% and a limiting sensitivity of about 10 ng/ml. A standard curve was prepared in the concentration range from 100 ng/ml to 25 $\mu\text{g/ml}$ (Fig. 11), and a good correlation was obtained between the plasma pimarinin concentration and peak AUC value ("AUC" refers to the area under the curve measurement that one gets as the exact reading from the fluorescence detector. I can be translated to drug concentration using a standard curve:

$$\text{AUC} = 1.2209e+4 + 3.2994e+5x, \quad r^2 = 1.00. \quad (\text{Eq. 1})$$

where e is the exponential function, x is the drug concentration that is sought, and r^2 is the correlation coefficient for the linear regression analysis for the ideal curve obtained from the actual data points in the observation interval.

The *in vivo* peak plasma pimarinin concentrations after iv administration of the above formulation was plotted for the two dose levels at the end of the 1 hour infusion and 5 hours later (Fig. 12); the measured concentrations are all within the *in vitro* range of sensitivity for the majority of the examined fungal isolates (see Tables 2 and 3).

Animal Experiment.

There were no clinically discernible cardiac arrhythmias assessed through clinical monitoring and serial EKGs before, during, and following the pimarinin infusions, and neither was there any detected impairment of hepatic or renal function over the 14-day experiment (Table 5). Group A consisted of two dogs (1 and 2) which were dosed at 1.0 and 5.0 mg/kg/day, respectively. Group B consisted of two dogs (3 and 4) which were also dosed at 1.0 and 5.0 mg/kg/day, respectively. Doses were administered to Group A on days 1-14 and to Group B on days 2-15. Samples were taken from Group A on day 0 (the day before treatment started), day 8 (after the first seven daily injections but before the eighth), and day 15 (the day after the final treatment). Samples were taken from

- 1 Group B on day 1 (the day before treatment started), day 9 (after the first seven daily
- 2 injections but before the eighth), and day 16 (the day after the final treatment).

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Table 5
Serum chemistry values in beagles after daily intravenous infusions of Pimaricin over a 14-day period

Table 5A
Day 0 or Day 1 (baseline)

| Group | Dog | Dose | Na | K | Cl | BUN | Creat | P | TP | Albu | DB | LDH | AST | ALT | TB | AP | GGT | Mg |
|-------|-----|------|-----|-----|-----|-----|-------|-----|-----|------|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 1 | 1.0 | 139 | 4.5 | 105 | 13 | 0.8 | 3.9 | 5.9 | 3.4 | 0.0 | 518 | 84 | 52 | 0.3 | 195 | 1 | 1.3 |
| A | 2 | 5.0 | 142 | 4.6 | 107 | 14 | 1.4 | 3.0 | 6.0 | 3.5 | 0.0 | 247 | 36 | 31 | 0.2 | 84 | 2 | 1.2 |
| B | 3 | 1.0 | 148 | 5.7 | 112 | 21 | 1.2 | 4.8 | 6.5 | 3.9 | 0.0 | 467 | 40 | 38 | 0.2 | 88 | 1 | 1.8 |
| B | 4 | 5.0 | 146 | 4.7 | 109 | 15 | 1.1 | 3.6 | 6.6 | 3.7 | 0.0 | 196 | 33 | 68 | 0.3 | 261 | 2 | 1.7 |

Table 5B
Day 8 or Day 9

| Group | Dog | Dose | Na | K | Cl | BUN | Creat | P | TP | Albu | DB | LDH | AST | ALT | TB | AP | GGT | Mg |
|-------|-----|------|-----|-----|-----|-----|-------|-----|-----|------|-----|-----|-----|-----|-----|------|-----|-----|
| A | 1 | 1.0 | 143 | 4.9 | 110 | 18 | 0.7 | 3.8 | 5.6 | 3.0 | 0.0 | 597 | 63 | 32 | 0.1 | 174 | 5 | 1.9 |
| A | 2 | 5.0 | 122 | 5.9 | 75 | 90 | 1.8 | 6.4 | 8.2 | 3.9 | 0.2 | 624 | 122 | 98 | 0.5 | 1093 | 12 | 3.4 |
| B | 3 | 1.0 | 135 | 4.9 | 103 | 20 | 0.9 | 3.7 | 5.6 | 3.3 | 0.0 | 675 | 54 | 37 | 0.2 | 80 | 3 | 1.9 |
| B | 4 | 5.0 | 132 | 3.8 | 98 | 24 | 0.9 | 3.0 | 5.5 | 2.7 | 0.1 | 527 | 59 | 232 | 0.2 | 911 | 15 | 1.7 |

Table 5C
Study termination (day 15 or day 16)

| Group | Dog | Dose | Na | K | Cl | BUN | Creat | P | TP | Albu | DB | LDH | AST | ALT | TB | AP | GGT | Mg |
|-------|-----|------|-----|------|-----|-----|-------|------|-----|------|-----|-----|-----|-----|-----|-----|-----|-----|
| A | 1 | 1.0 | 143 | 5.1 | 113 | 20 | 0.8 | 3.6 | 5.4 | 3.1 | 0.0 | 345 | 54 | 33 | 0.2 | 109 | 3 | 1.8 |
| A | 2 | 5.0 | 111 | 10.3 | 71 | 247 | 3.5 | 17.5 | 8.6 | 4.2 | -- | 751 | 545 | 205 | 1.0 | 625 | -- | -- |
| B | 3 | 1.0 | 144 | 5.2 | 111 | 20 | 1.1 | 4.6 | 5.5 | 3.5 | 0.0 | 211 | 33 | 34 | 0.1 | 56 | 3 | 2.0 |
| B | 4 | 5.0 | 144 | 4.0 | 108 | 21 | 0.9 | 3.2 | 5.3 | 2.9 | 0.0 | 63 | 26 | 64 | 0.2 | 424 | 8 | 1.6 |

1 Animal 2 died on day 12 of the study. Blood was obtained and analyzed, with the
2 exception of levels listed as (--), immediately post-mortem. Abbreviations used in the
3 table have the following meanings. Magnesium level indicated for animal 4 on day 9 is
4 the average of two readings.

| | | |
|----|-------|----------------------------------|
| 5 | Na | sodium |
| 6 | K | potassium |
| 7 | Cl | chloride |
| 8 | BUN | blood urea nitrogen |
| 9 | Creat | creatinine |
| 10 | P | phosphorus |
| 11 | TP | total protein |
| 12 | Albu | albumin |
| 13 | DB | direct bilirubin |
| 14 | LDH | lactic dehydrogenase |
| 15 | AST | serum aspartate aminotransferase |
| 16 | ALT | serum alanine aminotransferase |
| 17 | TB | total bilirubin |
| 18 | AP | alkaline phosphatase |
| 19 | GGT | gamma glutamyl transpeptidase |
| 20 | Mg | Magnesium |

21

Table 6
Hematologic values in beagles after daily intravenous infusions of Pimaricin over a 14-day period

Table 6A
Day 0 or Day 1 (baseline)

| Group | Dog | Dose | PT | PTT | Fibr | FDP | RET | WBC | HGB | HCT | MCV | PLT | Neu | Lym | Mon | Eos | Baso |
|-------|-----|------|-----|------|------|-----|------|------|------|------|------|-----|------|------|------|-----|------|
| A | 1 | 1.0 | 6.0 | 13.5 | 420 | neg | 0.4% | 39.1 | 12.8 | 37.3 | 68.0 | 246 | 92.2 | 3.1 | 4.1 | 0.2 | 0.0 |
| A | 2 | 5.0 | 5.5 | 13.3 | 190 | neg | 0.1% | 12.1 | 14.4 | 41.5 | 67.3 | 388 | 65.4 | 26.4 | 3.8 | 3.6 | 0.3 |
| B | 3 | 1.0 | 6.6 | 15.8 | 330 | neg | 0.7% | 12.9 | 16.4 | 49.1 | 69.9 | 544 | 73.5 | 18.8 | 4.9 | 2.4 | 0.2 |
| B | 4 | 5.0 | 5.5 | 15.3 | 420 | neg | 0.4% | 5.8 | 17.2 | 50.6 | 70.3 | 208 | 52.7 | 28.8 | 14.1 | 4.1 | 0.1 |

Table 6B
Day 8 or Day 9

| Group | Dog | Dose | PT | PTT | Fibr | FDP | RET | WBC | HGB | HCT | MCV | PLT | Neu | Lym | Mon | Eos | Baso |
|-------|-----|------|-----|------|------|-----|------|------|------|------|------|-----|------|------|------|-----|------|
| A | 1 | 1.0 | 6.0 | 12.3 | 390 | neg | 4.6% | 21.9 | 11.6 | 35.0 | 71.0 | 346 | 80.0 | 10.4 | 7.1 | 1.8 | 0.2 |
| A | 2 | 5.0 | 7.0 | 15.3 | 555 | neg | 2.8% | 48.8 | 18.6 | 53.0 | 64.1 | 112 | 89.0 | 5.4 | 4.2 | 0.7 | 0.1 |
| B | 3 | 1.0 | 5.3 | 12.8 | 230 | neg | 6.9% | 15.2 | 12.6 | 37.9 | 70.3 | 358 | 65.2 | 23.6 | 6.9 | 3.7 | 0.2 |
| B | 4 | 5.0 | 5.8 | 13.5 | 430 | neg | 7.2% | 16.2 | 13.6 | 40.5 | 71.6 | 138 | 75.4 | 11.9 | 10.3 | 1.8 | 0.1 |

Table 6C
Study termination (Day 15 or 16)

| Group | Dog | Dose | PT | PTT | Fibr | FDP | RET | WBC | HGB | HCT | MCV | PLT | Neu | Lym | Mon | Eos | Baso |
|-------|-----|------|------|------|------|-----|------|------|------|------|------|-----|------|------|-----|-----|------|
| A | 1 | 1.0 | 7.8 | 12.0 | 220 | neg | 2.1% | 14.2 | 11.0 | 33.4 | 72.4 | 255 | 80.0 | 14.0 | 3.9 | 1.8 | 0.1 |
| A | 2 | 5.0 | 10.8 | 23.5 | 330 | neg | 0.4% | 81.6 | 19.6 | 53.2 | 62.0 | 202 | 96.0 | 2.0 | 1.0 | 0 | 0 |
| B | 3 | 1.0 | 8.3 | 13.3 | 280 | neg | 0.9% | 14.5 | 12.3 | 37.0 | 71.3 | 421 | 65.4 | 25.2 | 4.4 | 4.6 | 0.1 |
| B | 4 | 5.0 | 8.3 | 13.0 | 310 | neg | 1.1% | 19.4 | 11.6 | 35.1 | 72.4 | 152 | 76.6 | 11.7 | 8.4 | 3.0 | 0.1 |

As mentioned above, animal 2 died on day 12 of the study. Blood was obtained and analyzed, with the exception of MCV which was calculated, immediately post-mortem. Abbreviations used in Table 6 have the following meanings.

| | |
|------|-----------------------------|
| PT | prothrombin time |
| PTT | partial thromboplastin time |
| Fibr | fibrinogen |
| FDP | fibrin degradation products |
| RET | reticulocytes |
| WBC | white blood cell count |
| HGB | hemoglobin |
| HCT | hematocrit |
| MCV | mean corpuscular volume |
| PLT | platelet count |
| Neu | neutrophils |
| Lym | lymphocytes |
| Mon | monocytes |
| Eos | Eosinophils |
| Baso | Basophils |

We found mild signs of hemolysis in the form of a gradual lowering of hemoglobin and hematocrit levels and a slight increase in reticulocyte counts during the study (Table 6). There was, however, no sign of bone marrow suppression/toxicity assessed by the white blood cell count, platelet count, or fibrinogen levels or any of the coagulation parameters (see Table 6). (Normal values for various hematological and serum chemistry parameters are provided in reference 44.)

Our data demonstrate the successful design of pharmaceutically acceptable formulations of pimarin, ones that are physiologically compatible with parenteral administration, with good tolerance and negligible toxicity, as demonstrated in the canine model. The intravenous infusion of one of the preparations in beagles provided plasma concentrations that reached and over many hours maintained fungicidal pimarin concentrations without any discernible untoward effects on the animals' clinical

1 performance or as detected by assessment of their hepatic or renal function during the 2-
2 week experiment. It should be noted, that for this experiment we selected the “fresh”
3 DMA/aqueous lipid formulation that had the highest concentration of an organic solvent,
4 DMA, to allow for the least favorable scenario when considering the potential for adverse
5 influence of the solvent system on hepatic and renal function, as well as on the
6 hematopoietic and cardiovascular systems.

7 Our data obtained with several diverse formulations demonstrate conclusively that
8 it should be feasible to introduce parenteral pimarinin in clinical therapy of systemic
9 fungal infections including fusariosis, with the predictable attainment of antibiotic
10 activity, and with a reasonable expectation of low normal organ toxicity. The inclusion
11 of a lyophilization step in the formulation procedure significantly increased the
12 stability/shelf-life of the final formulations. This step virtually eliminates the final
13 use-preparation’s content of the organic solvent, and we expect it not only to further
14 reduce the risk of solvent system toxicity, but also to minimize the risk that the organic
15 solvent could potentiate clinical adverse effects related to pimarinin.

16 It is apparent from the results that a dramatically improved bioavailability of
17 pimarinin was provided. Further, this novel preparation yielded plasma drug
18 concentrations and areas under the plasma concentration vs. time curves that were clearly
19 fungicidal, based on comparisons with our *in vitro* sensitivity studies with pimarinin
20 against several strains of *Aspergillus* spp., and *Candida* spp., but most importantly against
21 *Fusarium* spp., since this fungus is typically multidrug resistant. The present invention
22 makes it feasible to obtain beneficial effects of pimarinin against systemic mycoses, with
23 the potential for a major improvement in the outcome of such infections.

24 Compositions of the present invention can further include additional
25 pharmaceutically acceptable carriers, adjuvants, and/or biologically active substances.
26 Compositions of the present invention, as described above, can be used in methods for
27 treatment or prophylaxis of systemic fungal infections in mammals, particularly in
28 humans. The methods involve administering to a mammal an amount of the
29 compositions effective to prevent, eliminate, or control the fungal infection. The
30 administering step can suitably be parenteral (preferably by intravenous injection). The

1 compositions can also be administered intranasally as an aerosol. Such administration is
2 preferably repeated on a timed schedule, and may be used in conjunction with other
3 forms of therapy or prophylaxis, including methods involving administration of different
4 biologically active agents to the subject. The dose administered of a composition in
5 accordance with the present invention is preferably between approximately 0.1 and 100
6 mg/kg of body weight of the mammalian subject to which it is administered, most
7 preferably between about 1-5 mg/kg.

8 The preceding description of specific embodiments of the present invention is not
9 intended to be a complete list of every possible embodiment of the invention. Persons
10 skilled in this field will recognize that modifications can be made to the specific
11 embodiments described here that would be within the scope of the present invention.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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- 1 a pharmaceutically acceptable dipolar aprotic solvent; and
2 a pharmaceutically acceptable aqueous secondary solvent.
3
- 4 10. The method of claim 9, where the administration is intravascular.
5
- 6 11. The method of claim 9, where the aprotic solvent is N,N-dimethylacetamide.
7
- 8 12. The method of claim 9, where the aqueous secondary solvent is an aqueous lipid
9 emulsion.
10
- 11 13. The method of claim 12, where the aqueous lipid emulsion comprises a lipid
12 component that includes at least one vegetable oil and at least one fatty acid.
13
- 14 14. The method of claim 13, where the lipid component comprises at least about 5%
15 by weight soybean oil and at least about 50% by weight fatty acids.
16
- 17 15. The method of claim 9, where the secondary solvent is selected from the group
18 consisting of water, saline solution, and dextrose solution.
19
- 20 16. A method of preparing an antifungal composition, comprising:
21 dissolving pimarinol or an antifungal derivative thereof in a pharmaceutically
22 acceptable dipolar aprotic solvent; and
23 adding to the solution a pharmaceutically acceptable aqueous secondary solvent.
24
- 25 17. The method of claim 16, further comprising the step of lyophilizing the
26 composition, whereby the majority of the aprotic solvent is removed from the
27 composition and a dry, shelf-stable composition is produced.
28
- 29 18. The method of claim 17, further comprising the step of reconstituting the dry
30 composition by the addition of a pharmaceutically acceptable aqueous solvent.

1 **WHAT IS CLAIMED IS:**

2 1. An antifungal composition suitable for parenteral administration to a mammal,
3 comprising:

4 an amount of pimarinin or an antifungal derivative thereof that is effective to
5 inhibit the growth of a systemic infection in a mammal;
6 a pharmaceutically acceptable dipolar aprotic solvent; and
7 a pharmaceutically acceptable aqueous secondary solvent.

8
9 2. The composition of claim 1, where the aprotic solvent is N,N-dimethylacetamide.

10
11 3. The composition of claim 1, where the aqueous secondary solvent is an aqueous
12 lipid emulsion.

13
14 4. The composition of claim 3, where the aqueous lipid emulsion comprises a lipid
15 component that includes at least one vegetable oil and at least one fatty acid.

16
17 5. The composition of claim 4, where the lipid component comprises at least about
18 5% by weight soybean oil and at least about 50% by weight fatty acids.

19
20 6. The composition of claim 1, where the secondary solvent is water.

21
22 7. The composition of claim 1, where the secondary solvent is saline solution.

23
24 8. The composition of claim 1, where the secondary solvent is dextrose solution.

25
26 9. A method of preventing or treating a systemic infection in a mammal, comprising
27 administering parenterally to a mammal a composition that comprises:

28 an amount of pimarinin or an antifungal derivative thereof that is effective to
29 inhibit the growth of an infectious agent that is sensitive to pimarinin in
30 the mammal;

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19. The method of claim 18, where the pharmaceutically acceptable aqueous solvent is selected from the group consisting of water, saline solution, and dextrose solution.

20. The method of claim 16, where the aprotic solvent is N,N-dimethylacetamide.

21. The method of claim 16, where the aqueous secondary solvent is an aqueous lipid emulsion.

22. The method of claim 21, where the aqueous lipid emulsion comprises a lipid component that includes at least one vegetable oil and at least one fatty acid.

23. The method of claim 22, where the lipid component comprises at least about 5% by weight soybean oil and at least about 50% by weight fatty acids.

ABSTRACT

An antifungal composition suitable for parenteral administration to a mammal includes an amount of pimarinic acid or an antifungal derivative thereof that is effective to inhibit the growth of a fungal infection in a mammal; a pharmaceutically acceptable dipolar aprotic solvent; and a pharmaceutically acceptable aqueous secondary solvent. The composition can be used in methods of preventing or treating a systemic fungal infection in a mammal. The composition can be prepared by dissolving pimarinic acid or an antifungal derivative thereof in the pharmaceutically acceptable dipolar aprotic solvent; adding to the solution a pharmaceutically acceptable aqueous secondary solvent; and in a preferred method, by subsequently lyophilizing the composition, whereby a dry, shelf-stable composition is produced. This dry composition can be reconstituted into an aqueous solution suitable for parenteral administration.

1

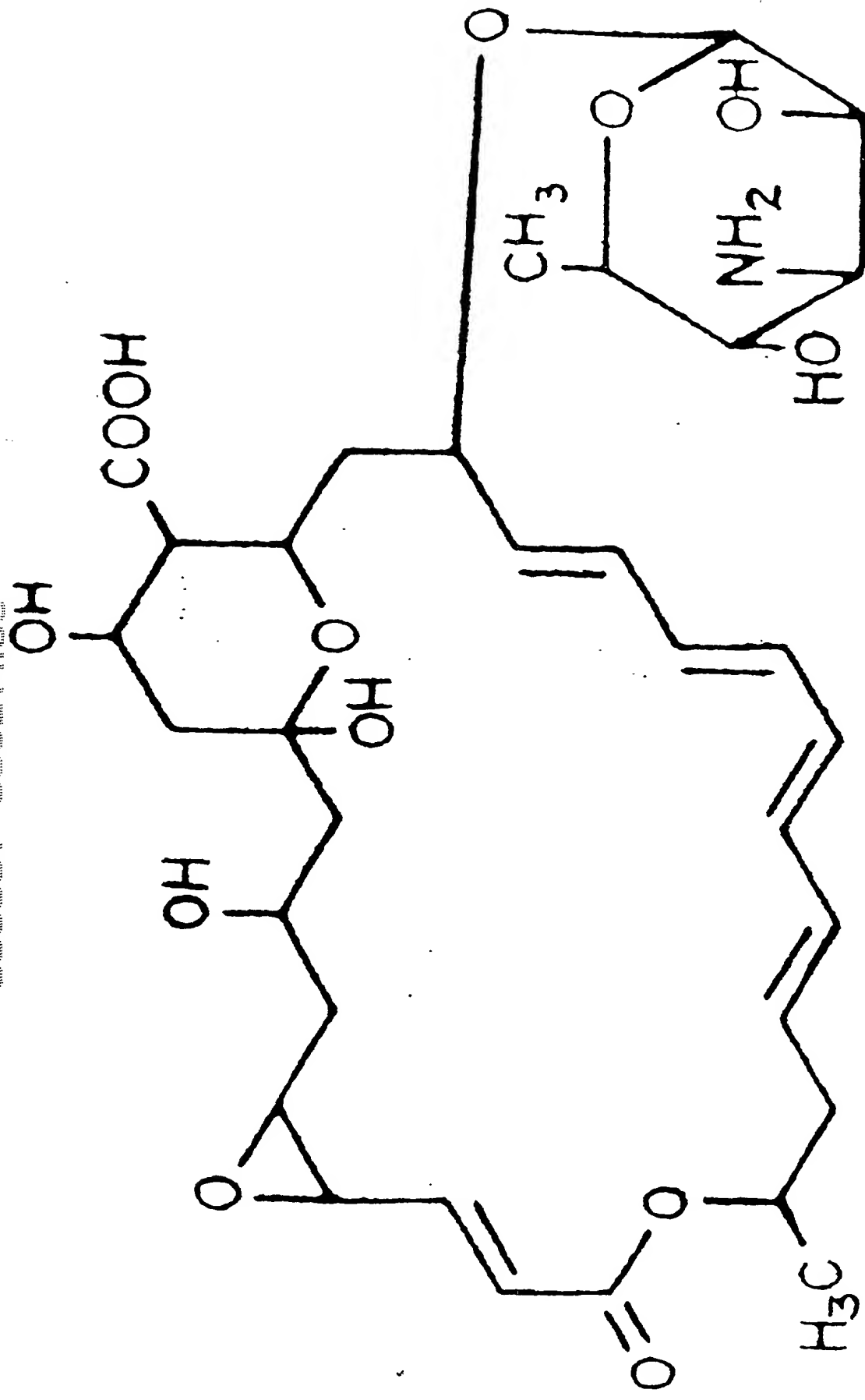


Fig. 1

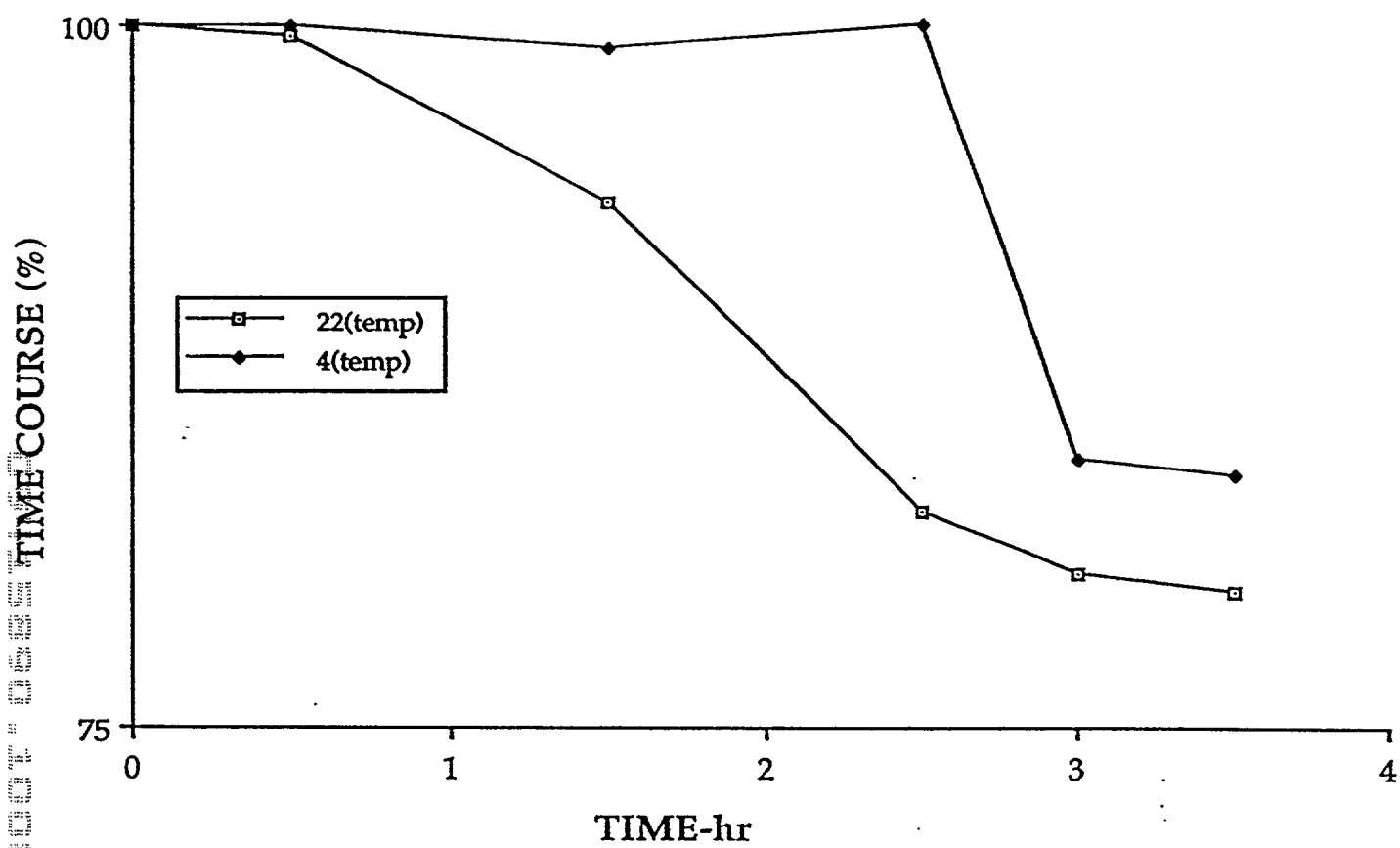


Fig. 2

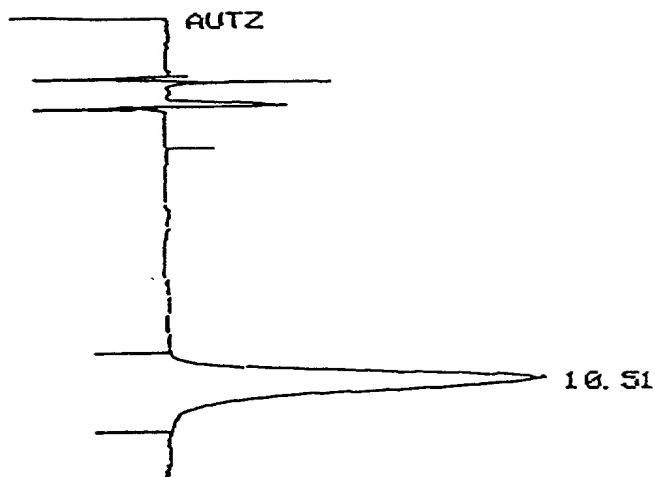


Fig. 3A

RUN TIME 13.25

DEFAULT 0
NORMALIZATION METHOD USING AREA

| TIME | AREA | %AREA | PK NAME |
|-------|---------|-------------|---------|
| 10.51 | 1411653 | 100.0000000 | |
| TOTAL | 1411653 | 100.0000000 | |

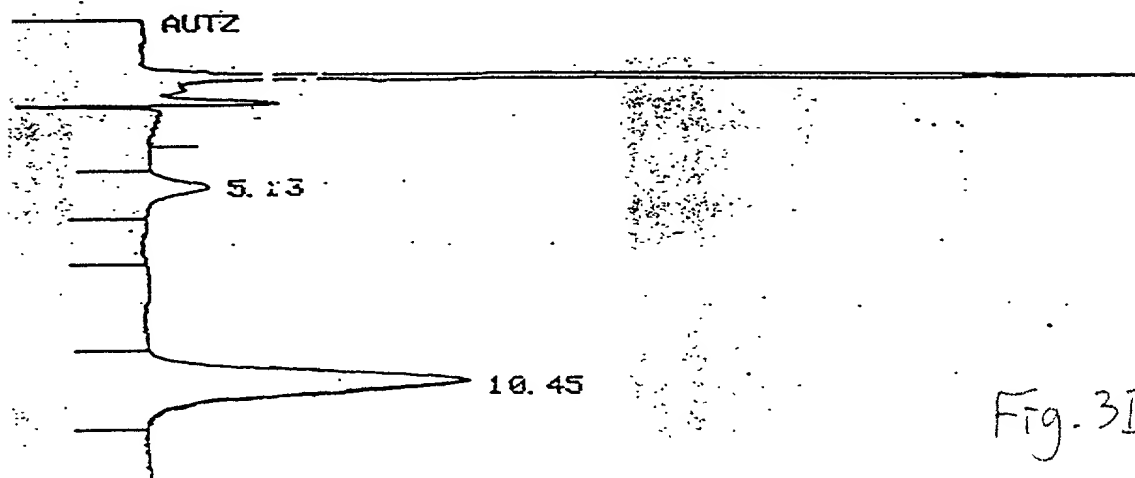


Fig. 3B

RUN TIME 10

DEFAULT 0
NORMALIZATION METHOD USING AREA

| TIME | AREA | %AREA | PK NAME |
|-------|---------|-------------|---------|
| 5.13 | 121808 | 9.142218966 | |
| 10.45 | 1210568 | 90.85778103 | |
| TOTAL | 1332368 | 100.0000000 | |

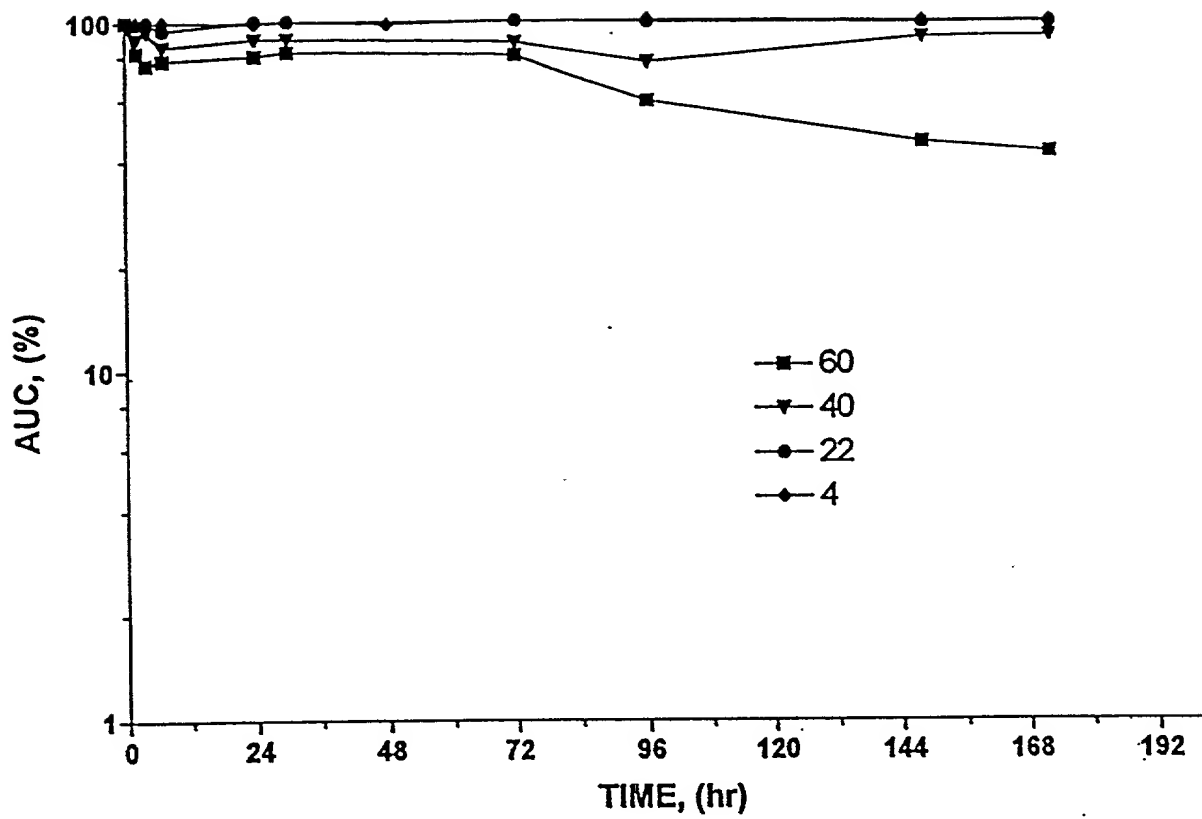


Fig. 4

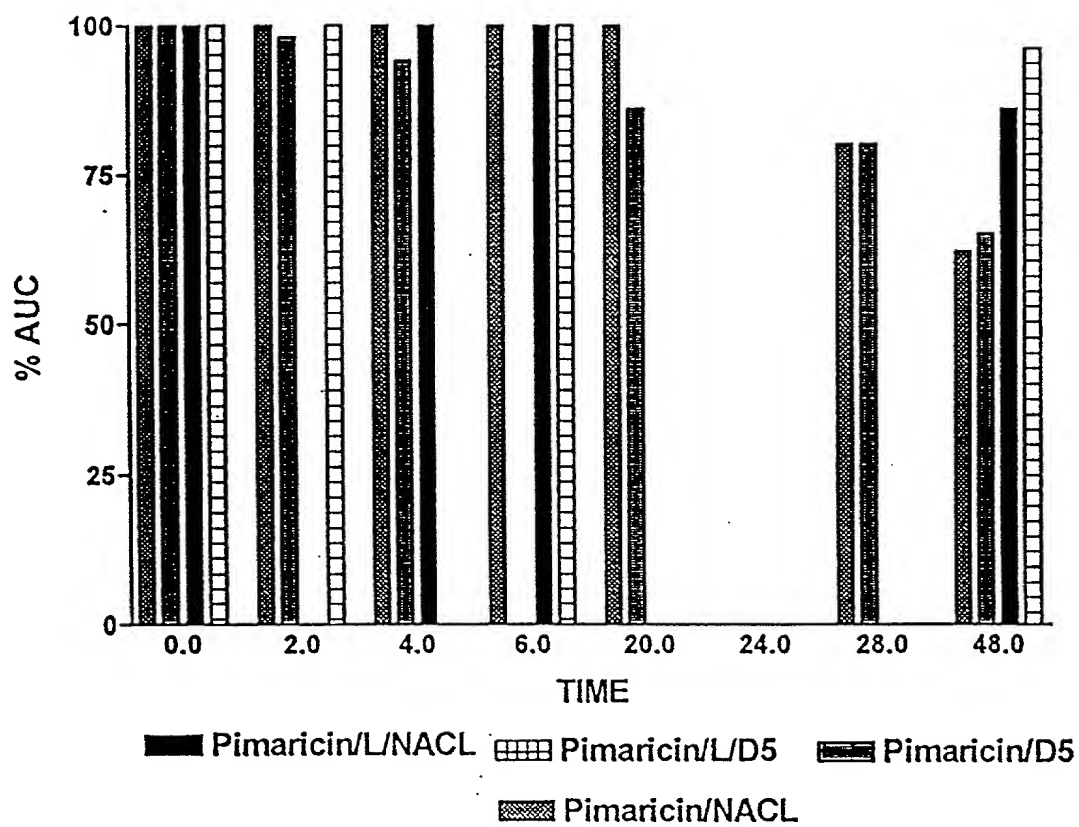
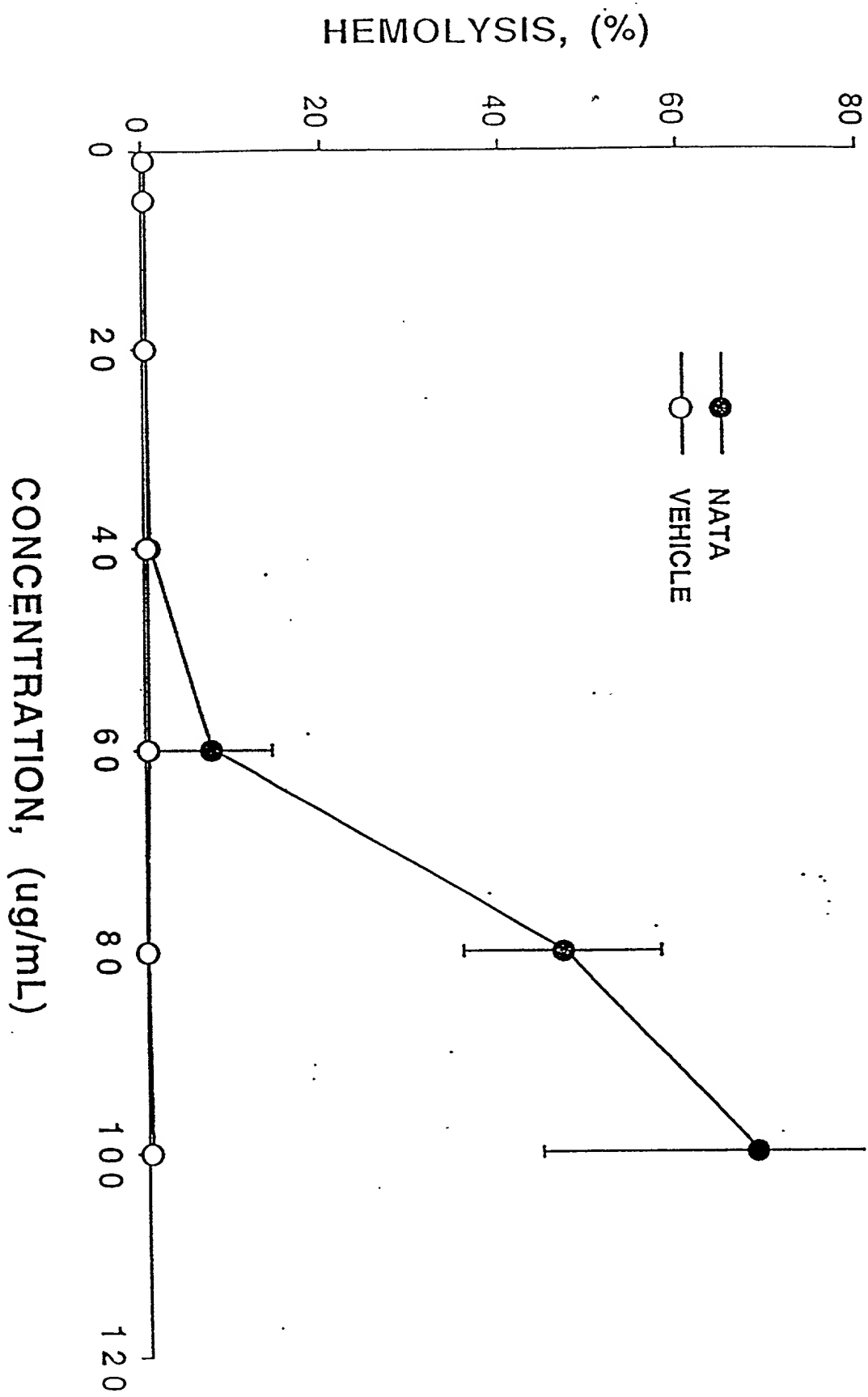


Fig. 5

Fig. 6



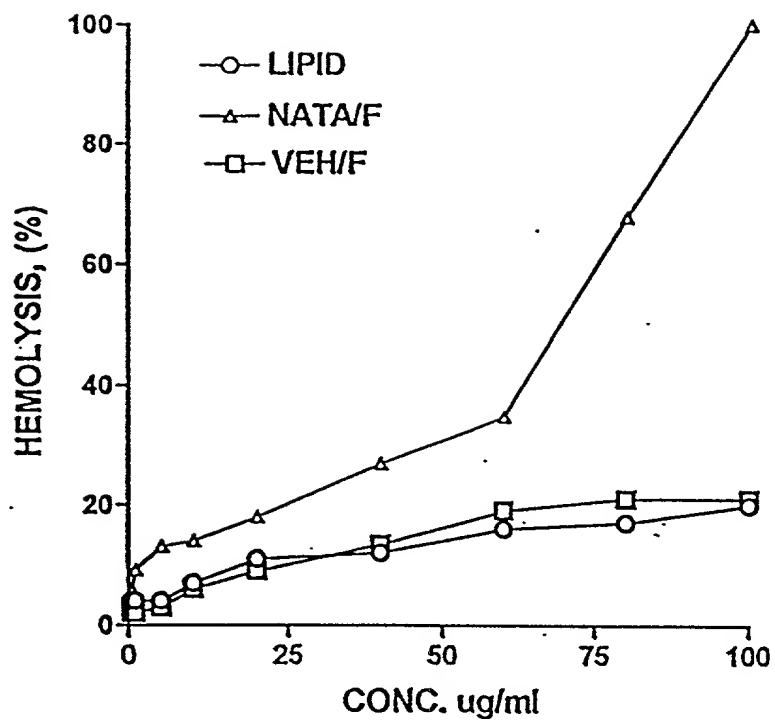


Fig. 7

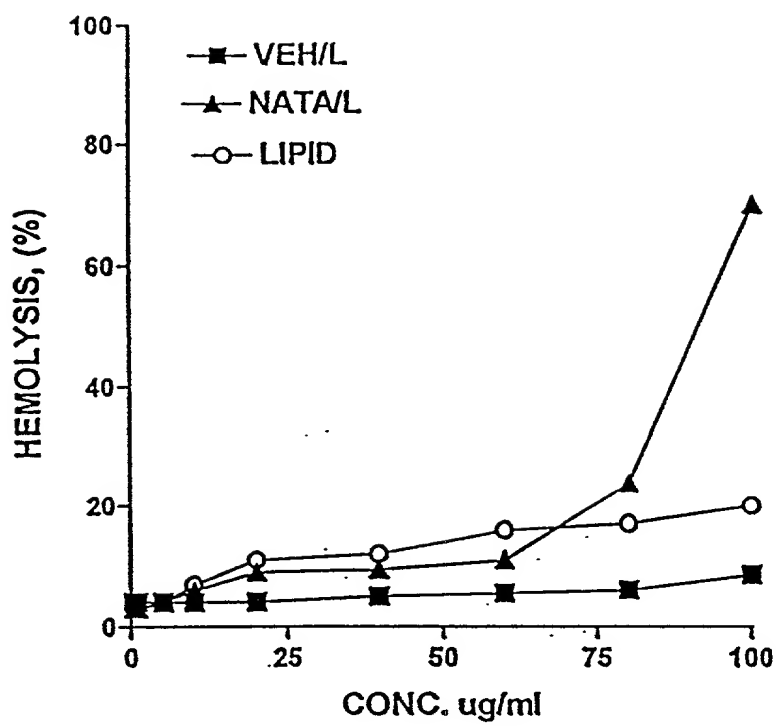


Fig. 8

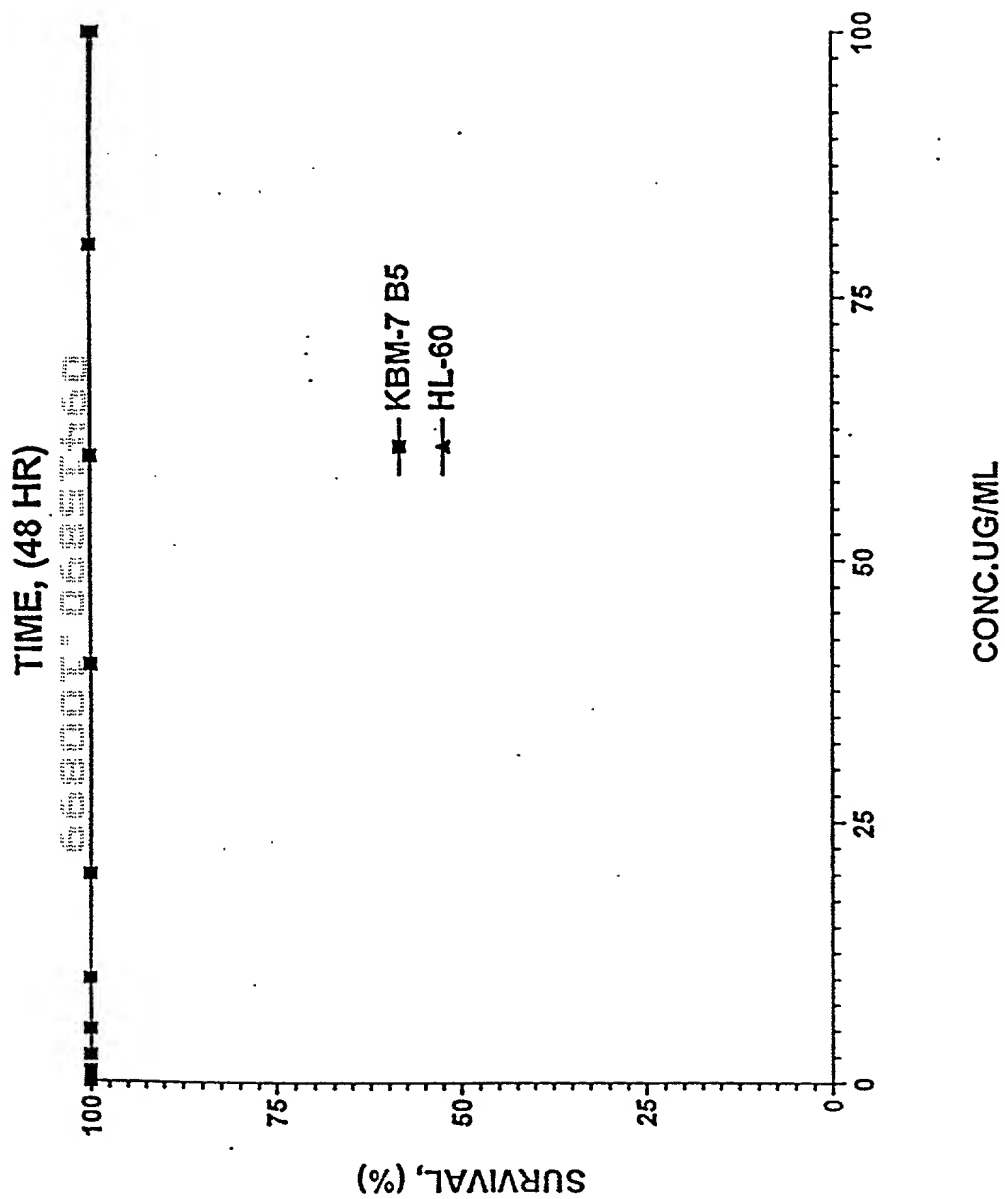


Fig. 9A

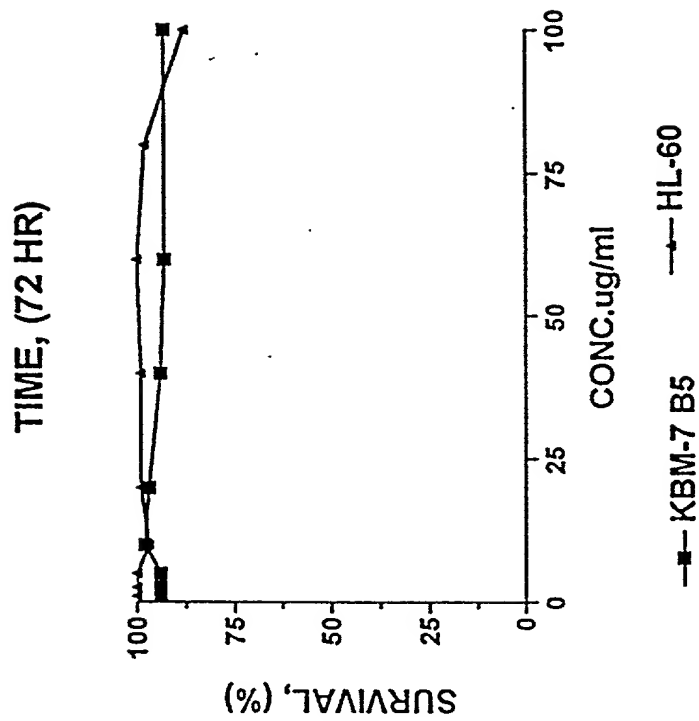


Fig. 9B

AUTZ

12.81
13.76
14.01

Fig. 10A

RUN TIME 14.50

NO PEAKS FOUND

AUTZ

5.28

10.03

12.22 12.38

13.07

13.64 13.78

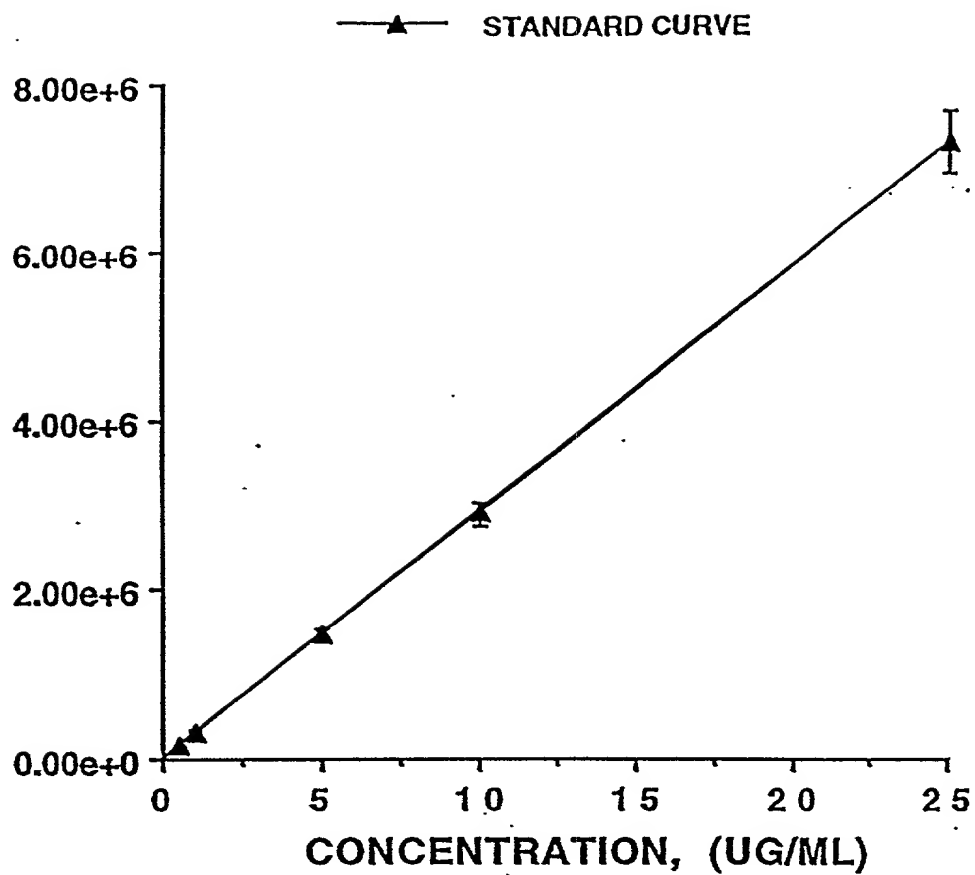
RUN TIME 14.50

FIG. 10B

DEFAULT 0
NORMALIZATION METHOD USING AREA

| TIME | AREA | ZAREA | PK NAME |
|-------|---------|-------------|---------|
| 5.28 | 44380 | 3.879435060 | |
| 10.03 | 1039601 | 96.12056493 | P |
| TOTAL | 1143981 | 100.0000000 | |

AUC



Curve fit = $y = -557.10 + 2.9201e+5x$ $R^2 = 1.000$

Fig. 11

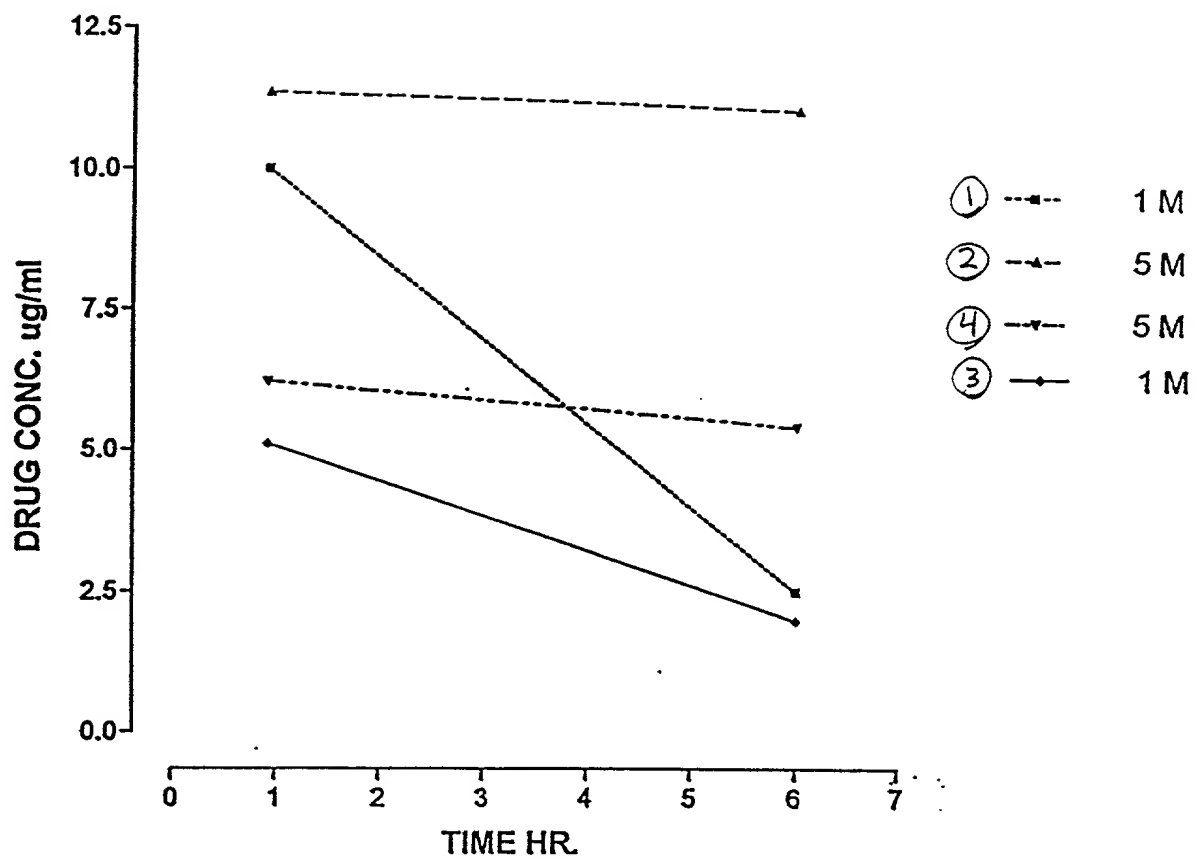


Fig. 12

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: §
Borje S. Andersson §
Serial No.: Unknown § Examiner: Unknown
Filed: October 8, 1999 § Group Art Unit: Unknown
For: PHARMACOLOGICALLY § Atty. Dkt.: UTXC:528--1/PAR
ACCEPTABLE SOLVENT VEHICLES §
§

JC675 U.S. PTO
09/415890
10/08/99

| | |
|--------------------------------|-----------------|
| CERTIFICATE OF EXPRESS MAILING | |
| NUMBER | EL258326531US |
| DATE OF DEPOSIT | October 8, 1999 |

STATEMENT REQUESTING DELETION OF AN INVENTOR
UNDER 37 CFR § 1.63(d)(2) and 1.33(b)

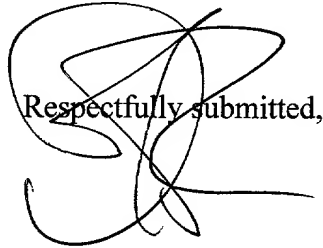
Sir:

Co-pending U.S. Patent Application Serial No. 08/911,607 entitled "PARENTERAL PIMARICIN AS TREATMENT OF SYSTEMIC INFECTIONS" was filed on August 15, 1997, properly naming as inventors, Borje S. Andersson and Elias J. Anaissie. The above captioned continuation Patent Application claiming priority to co-pending U.S. Patent Application Serial No. 08/911,607 is being filed herewith this paper. Also being filed herewith is a Preliminary Amendment canceling the claims and filing new claims for the present Patent Application.

Elias J. Anaissie is no longer an inventor of a claim remaining in the present Patent Application. It is respectfully requested that Elias J. Anaissie be deleted as an inventor in the captioned Patent Application.

No fees are believed to be due in connection with the filing of this Statement Requesting Deletion of an Inventor, however, should any fees under 37 C.F.R. §§ 1.16 to 1.21 be deemed

necessary for any reason relating to these materials, the Assistant Commissioner is hereby authorized to deduct said fees from Arnold White & Durkee Deposit Account No. 01-2508/UTXC:528--1/PAR.

 Respectfully submitted,

David L. Parker
Reg. No. 32,165

ARNOLD, WHITE & DURKEE
P.O. Box 4433
Houston, Texas 77210
512-418-3000

Attorney for Applicants

Date: October 8, 1999

DECLARATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or the below named inventors are the original, first and joint inventors (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **Parenteral Pimaricin as Treatment of Systemic Infections**, the Specification of which:

- ☒ is attached hereto.
☐ was filed on _____ as Application Serial No. _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims.

I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability of the subject matter claimed in this application, as "materiality" is defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

| PRIOR FOREIGN APPLICATION(S) | | | Priority Claimed |
|------------------------------|-----------|--------------|---------------------|
| (Number) | (Country) | (Date Filed) | Yes/No |
| (Number) | (Country) | (Date Filed) | Yes/No |

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose all information known to me to be material to patentability of the subject matter claimed in this application, as "materiality" is defined in Title 37, Code of Federal Regulations, § 1.56, which become available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.) (Filing Date) (Status)

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I hereby declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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